Don Wicklow

AFLATOXIN ELIMINATION WORKSHOP

MEMPHIS, TN
October 26 - 28, 1997

A Decade of Research Progress
1988 - 1997

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AFLATOXIN ELIMINATION WORKSHOP

Memphis, Tennessee
October 27-28, 1997

Throughout the world, aflatoxin is considered one of the most serious food safety problems. Chronic problems with aflatoxin contamination occur in the southern U.S. in cottonseed, corn, peanuts and tree nuts. However, the impact of aflatoxin contamination on the agricultural economy is especially devastating during drought years when aflatoxin affects the Midwestern corn belt. Estimated economic losses in years of major aflatoxin outbreaks have been in the hundreds of millions of dollars.

The Aflatoxin Elimination Workshops have served to bring together the Agricultural Research Service (ARS, USDA), university scientists, and representatives of the cottonseed, corn, peanut, and tree nut industries in a unique cooperative effort to develop aflatoxin control strategies through research and development. The ultimate goal of this effort is to facilitate the commercial implementation of technologies to eliminate the aflatoxin contamination problem in the U.S. marketplace by the turn of the millennium. Most of the research to eliminate aflatoxin is conducted by ARS; however, an important addition to this effort is a competitive award program provided by Congressional appropriations to bring to the research effort the talents of university scientists in cooperation with ARS.

Scientists realized over a decade ago that the aflatoxin problem could not be solved solely by conventional technologies utilized routinely to control the more "typical" plant pathogens. Aflatoxin contamination is a result of fungal infection of host plants by a unique class of microorganisms adapted to subsist saprophytically on organic debris in the field or to infect and produce aflatoxin in living plant tissues. Classical plant disease prevention methods developed to control very fastidious plant pathogens have been generally unsuccessful in excluding aflatoxin producing fungi from their relatively broad ecological niche. The realization of the unique nature of the aflatoxin problem and that novel technologies will be required for its control became a focal point of discussion during strategy development sessions of the first Aflatoxin Elimination workshop in 1988. Two areas of research and development based on the biology and ecology of *Aspergillus flavus*-group fungi were suggested: 1) novel genetic engineering and/or marker-based breeding methods to enhance general antifungal resistance in crops, and 2) the isolation and formulation of special fungi for use in biocontrol. These biocontrol fungi are strains of *A. flavus*-group fungi that do not produce aflatoxin, but have the capability to occupy the same ecological niche in the field and out compete harmful toxin-producing fungi.

The vision of participants in the first Aflatoxin Elimination Workshop has been confirmed by the rapid progress reported in subsequent workshops in developing practical, commercially viable aflatoxin control procedures, based almost entirely on the concepts generally established during the first workshop. In the area of plant breeding, aflatoxin gene regulation, and use of native plant compounds against *A. flavus*, notable success has been made in the development and testing of "reporter" strains of aflatoxin-producing fungi. Recent experiments involving both laboratory and field infection of corn kernels by reporter strains of *A. flavus* have allowed quantitation of the amount of fungal invasion in kernels, a critical measurement in the assessment of resistance during corn varietal screening by plant breeders. The reporter strains are also being used to detect compounds in corn kernels that modulate aflatoxin production.

This has been made possible through identification of the regulatory loci of genes governing aflatoxin formation, which were adapted for use in production of reporter gene containing strains. In addition, progress has been made in the identification of several plant modulators of *A. flavus* growth/aflatoxin production. Collaboration between university and ARS scientists involving the use of reporter fungi and inhibitory compounds has generated great interest in the corn-breeding community, which is using these tools to select for resistance.

Advancements have been made in the field of genetic engineering, particularly in cotton where cotton embryo transformation with antifungal genes has been accomplished. Through cooperation between ARS and university scientists, it has also become possible to achieve germline transformation of walnut and peanut with antifungal genes with the goal of enhancing resistance to *A. flavus* in these crops.

Further success was seen in the area of biological control where continued large-scale testing under the EPA Experimental Use Permit in Arizona resulted in reduced infection of cottonseed by toxigenic strains and reduced aflatoxin levels. Experiments in Georgia showed for the first time that biological control could have an impact on aflatoxin contamination of corn. Aflatoxin levels were reduced in corn that was treated with atoxigenic strains of *A. flavus* and *A. parasiticus* during 1996 and 1997. In addition, field-grown peanuts from the 1997 drought-stressed crop that had been treated with the biocompetitive strains had aflatoxin levels reduced by 90% compared with untreated peanuts.

Although biologically-based technologies are important themes of the workshops, improvement of crop management and handling practices has also been emphasized. Based on greater understanding of the biology and ecology of insect and fungal-plant interactions, the development of cultural practices used in control of insects and aflatoxin in tree nuts has been possible. The workshop also reinforced the need to coordinate such crop management and handling practices as optimum time of harvest, irrigation, insect control, and mechanical exclusion of bad seed/kernels to eliminate aflatoxin in the final food or feed product. These careful crop management/handling practices will probably always be important even with the use of powerful new biologically-based technologies.

Research information summarized in this 1997 workshop proceedings provides the technological foundation for the multiple strategies currently being investigated to eliminate aflatoxin, thus leading to the well grounded optimism that solutions to this serious food safety problem will be available by the beginning of the next century. This innovative aflatoxin control technology under development was made possible only by the ingenious application of research information on the nature of *A. flavus*-group fungi and of the aflatoxin contamination process, knowledge which has been provided over the years by scientists attending the workshop.

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Thanks are due to Deepak Bhatnagar and Ed Cleveland for organizing the workshop agenda.

The Session and Panel Chairs are commended for their leadership in facilitating the scientific program and panel discussions.

Appreciation is extended to Linda Deer, Althea Hunt, Deepak Bhatnagar, Pat Deshotel and Ed Cleveland of the Southern Regional Research Center, New Orleans, Louisiana, for compilation and editing of the Workshop Proceedings.

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AFLATOXIN ELIMINATION WORKSHOPS

New Orleans, LA	1988
Peoria, IL	1989
St. Louis, MO	1990
Atlanta, GA	1991
Fresno, CA	1992
Little Rock, AR	1993
St. Louis, MO	1994
Atlanta, GA	1995
Fresno, CA	1996
Memphis, TN	1997
St. Louis, MO	1998

COOPERATING COMMODITY GROUPS

Peanuts:	American Peanut Council
Corn:	American Corn Millers Federation National Corn Growers Association Corn Refiners Association
Cottonseed:	National Cottonseed Products Association National Cotton Council
Tree Nuts:	Almond Board of California California Pistachio Commission Dry Fruits Association of California Walnut Marketing Board

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CROP MANAGEMENT AND HANDLING, INSECT CONTROL AND FUNGAL RELATIONSHIPS

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Session 1

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PANEL DISCUSSION

PANEL DISCUSSION TITLE: Crop Management and Handling, Insect Control and Fungal Relationships

PANEL MEMBERS: Pat Dowd (Chair), Peter Cotty, Tom Isakeit, Themis Michailides, Tom Pearson and Paul Williams

SUMMARY OF PRESENTATIONS: Plant stress, both in the field and storage, continue to be identified as factors important in determining aflatoxin levels in different commodities. Insects can contribute to this stress, as well as vector or cause damage conducive to the invasion of mycotoxigenic fungi. For example, early boll damage by insects is associated with increased levels of aflatoxin (Isakeit, Cotty et al.), but delayed harvesting and prolonged storage of modules can be as, or more important (Bock and Cotty). Drought tolerance of peanuts (Basha) and irrigation of pistachios to prevent early splits and of figs to prevent drought stress (Michailides et al.) Can potentially reduce aflatoxin levels in these crops. Subsurface drip irrigation (vs. surface drip) helps figs dry faster and reduces the levels of molds in drying figs. Bt corn (Dowd et al., Williams et al.) and cotton (Cotty et al.) are being tested for indirect decreases in mycotoxins through; control of insect damage, with mixed results. Natural insect resistance factors in corn (Dowd et al.) and proper storage that decreases insects in Nigeria and Benin (Cardwell et al.) should reduce mycotoxins. Sorting of first crop, "long necked" figs that often have higher levels of aflatoxin, and sorting of abnormal tree nuts through light imaging (Pearson et al.) or x-ray imaging (Casasent et al, Keagy and Miller) can also reduce aflatoxin in these commodities.

SUMMARY OF PANEL DISCUSSION: In response to a request from Dowd, Will Duensing described a small density difference air separator that is being tried on corn to get rid of lower density insect/fungus damage kernels. Howard Valentine indicated peanut separation with the same method did not provide fine distinction of different levels of aflatoxin in contaminated peanuts. Pearson indicated that their image based feature extraction and use of discrimination analysis is more effective than other machines on the market, which are only able to extract one feature.

Dorner asked for information on the subsurface drip irrigation described by Michailides in terms of cost and benefits. Themis indicated the drier soil helps reduce all molds and helps the figs dry faster. The cost is high initially, but the quality of figs, and water saved, has made it cost effective enough that growers are adopting it. Doster also indicated it helps reduce weed problems. Cotty asked if subsurface irrigation acts by reducing sporulation of fungi on the soil surface or by direct effects on the host. Michailides indicated they believe this type of irrigation puts less stress on the trees, which is beneficial for peanuts and corn. He indicated the green fruit is very resistant, but stress may change the physiology to make it more susceptible. Doster indicated the situation is very complicated. The fig ostiole is often larger with more irrigation, which could potentially promote more internal contamination. Increased irrigation reduces incidences of fruit decay by fungi in Section *Flavi* and *Fusarium* (endosepsis), but not by Section *Nigri* or dermatiaceous ones. Michailides indicated dermatiaceious fungi included *Alternaria*, *Dendryphiella*, and *Ulocladium*, and that *Alternaria* toxins are not an issue yet.

Kitty Cardwell indicated European labeling requirements for genetically modified organisms had caused some problems when soybean oil imported from the U.S. that had been used in

some batches of candy bars ultimately resulted in all of the bars being taken off of the market because batches had not been segregated as to oil source. Pat Dowd indicated there appears to be variability in efficacy of Bt corn in indirectly reducing mycotoxigenic fungi, with generally good results being seen in the Midwest, but only limited effectiveness in the South. He wondered if this was a function of greater proportion of pest insects being the target insects (European corn borers) in the Midwest compared to the South, and asked Williams to comment. Williams indicated that in their trials in Mississippi, when silk inoculations were done (the method of choice in the Bt assays) vs. mechanical damage results can be variable from year to year. In some years they would get little aflatoxin, while in other years there would be high levels of aflatoxin in the absence of insects. Windham indicated when the inoculum is injected in the silk channel it usually works well, but when silks are sprayed usually there is little aflatoxin, even then the spray follows insect damage.

Cotty indicated the Bt cotton can be very effective in reducing aflatoxin in cottonseed when it is harvested early and processed promptly, but when allowed to sit while awaiting harvest, it can get as bad as non-Bt cotton. During cotton harvest, ginning initially proceeds rapidly. As the harvest progresses, the gins become backed up, and modules sit, potentially increasing in aflatoxin levels. Peter indicated there is some concern that growers may delay harvest with the Bt cotton because the insect control provided by the Bt cotton makes the cost of keeping the crop growing relatively low. This delay in harvest may result in increased aflatoxin levels. Peter indicated that *A. flavus* can colonize the lint, and that the possibility that infected lint is acting as an inoculum source in the modules is being investigated.

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TIMING OF AFLATOXIN CONTAMINATION OF COTTONSEED IN SOUTH TEXAS IN RELATION TO BOLL MATURITY

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The objective of this study was to identify factors associated with aflatoxin contamination of cottonseed over the course of the season, with particular emphasis on insect damage. Cotton samples were removed from modules stored in harvested fields and at gins located in San Patricio county. Locks showing visible insect injury were segregated from the other locks and the seed samples were analyzed separately. Seed associated with bright green yellow fluorescent (BGYF) lint, indicating *Aspergillus flavus* infection prior to boll maturity, was analyzed separately from seed originating from other, non-BGYF, insect-damaged locks.

Of the segregated samples with no visible damage, only 8% had high aflatoxin levels (range: 20-120 ppb). These samples had no BGYF seed associated with them and although they all had contained non-BGYF, damaged seed, the aflatoxin contents of these samples were low except for one (37,125 ppb).

Of the samples examined, 44% contained BGYF seed and 98% contained non-BGYF, damaged seed. Negligible aflatoxin concentrations were detected in 54% of BGYF seed samples, while the remainder exceeded 1,000 ppb. The highest aflatoxin concentrations of BGYF samples ranged from 177,500-302,200 ppb and represented 12% of the samples. Such highly-contaminated seed comprised 0.04% of the original samples and if they were to be added back to the non-damaged portion of the samples, they would result in overall aflatoxin concentrations of 75-124 ppb.

Neglible aflatoxin concentrations were detected in 48% of non-BGYF, damaged samples, while concentrations in the remainder ranged from 20-37,125 ppb. Samples that exceeded 2,000 ppb, which represented 9% of the total, comprised 0.5-2% of the total weight of original samples. If these contaminated seeds were to be added back to the non-damaged portion of the samples, they would result in overall concentrations of 30-187 ppb.

These results indicate a relationship of insect injury to aflatoxin contamination. However, they do not account for a late-season increase in contamination of seed shipments received from San Patricio county gins by a Harlingen oil mill. In 1996, weathering, caused by a long period of rain, could account for this increase in aflatoxin late in the season, but there was no such rainy period in the 1997 season.

AFLATOXIN CONTAMINATION IN COTTONSEED AT WEEKLY INTERVALS IN MODULES FROM ARIZONA, MISSISSIPPI AND TEXAS

W. E. Batson, Jr. and J. Caceres[1], P. J. Cotty[2] and T.Isakeit[3], [1]Department of Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA; [3]Texas Agricultural Research and Extension Center, Weslaco, TX

Two cotton modules were constructed near Odem, Texas, and two near Roll, Arizona from producer fields in areas with histories of aflatoxin problems. The Texas modules, TX-1 and TX-2, were constructed on August 13, 1996 from cotton plots comparing Ginstar/Cyclone and Harvade/Cyclone harvest-aid materials, respectively. The Arizona modules, AZ-1 and AZ-2, were constructed on September 25, 1996. Texas and Arizona modules were transported to the gin yard for storage. In addition, two modules, MS-1 and MS-2, were constructed at the Plant Sciences Research Center at Starkville, MS from fields infested at boll-crack with 7 lbs./A of wheat kernels colonized with a highly toxigenic isolate of *A. flavus*. These modules remained in the field throughout the study. Module sampling ports (MSP) were installed within all modules during construction to permit access to the module interior for repeated sampling. Samples were taken from each sampling port at module construction and weekly for three weeks for the Texas modules and four weeks for the Arizona and Mississippi modules. Also, two samples were taken weekly from the exterior of each module. Samples from Texas and Arizona were shipped overnight to Mississippi for aflatoxin analysis.

Mean aflatoxin concentration of composite samples composed of cottonseed meal from all sampling ports of TX-1, ranged from 0 ppb at the time of moduling to 43 ppb after three weeks of storage. Regression analysis of aflatoxin levels versus time was positive and significant indicating that aflatoxin increased over time in the TX-1 module. Levels of aflatoxin were lower in TX-2 and regression of aflatoxin level on time was negative indicating that levels decreased over time. Temperatures within TX-2 were higher than in TX-1 and increased over time to above 40 C. Temperatures recorded within TX-1 were always below 38 C and decreased with time. Maximal production of aflatoxin in cottonseed has been reported to occur between 25 - 30 C. Mean aflatoxin levels for MS-1 ranged from 0 ppb at moduling to 23 ppb after four weeks of storage and increased weekly throughout the study. Regression analysis of aflatoxin level versus time was significant for MS-1. Levels of aflatoxin in composite samples from AZ-1 and AZ-2 were generally low and remained below threshold levels throughout the study. Modules in the Arizona study area constructed at the same time as ours were seldom rejected because of aflatoxin problems. However, aflatoxin problems did develop in modules constructed toward the end of the season in Arizona. The relationship of aflatoxin level and storage time in AZ-1, AZ-2, and MS-2 was also positive but not significant.

GROWER PRACTICES THAT REDUCE AFLATOXIN CONTAMINATION OF PISTACHIOS AND FIGS

M. A. Doster, T. J. Michailides, and D. P. Morgan, Dept. of Plant Pathology, and
D. A. Goldhamer, Dept. of Land, Air & Water Resources, University of California,
Davis/Kearney Agricultural Center, Parlier, CA

Pistachios. The evaluation of nut samples harvested at three different dates in 1996 was completed. The incidence of fungal decay in early splits increased only slightly from 75% for the first harvest to 87% for the last harvest 20 days later, while the incidence in nuts with cracked hulls increased substantially from 26 to 59%. For all types of nuts considered together, fungal decay approximately doubled from the first harvest to the last. For nuts with cracked hulls and for early splits, the nuts with shriveled hulls had substantially higher incidences of decayed kernels than nuts with smooth hulls. A similar experiment with three harvest dates was performed in 1997, and the nut samples are currently being evaluated.

Studies were continued with chemicals that induce pistachio trees to break dormancy early. In one study, hydrogen cyanamide (sprayed 6 Feb.) increased the percentage of early splits at harvest (0.9 and 0.5% for sprayed and controls, respectively). In another experiment, a single application of Volck oil sprayed on 13 March (11.2% early splits at harvest time) or 17 January (10.7%) increased the formation of early splits compared to the nonsprayed control (4.4%) and to spraying Volck oil on 13 February (4.2%). Therefore, pistachio growers should pay special attention when to apply these chemicals that induce an early breaking of bud dormancy.

The effect of removing nuts left on the tree after harvest on navel orangeworm (NOW) infestation (highly associated with aflatoxin contamination) has been determined for the 1996 and 1997 harvests. In 1996 trees that had nuts left on them (from the previous year) had approximately twice the incidence NOW infestation at harvest as trees that had nuts removed during the previous winter. In 1997 when nuts were removed from the entire orchard during winter, the incidence of NOW infestation was approximately the same throughout the orchard except near an adjoining almond orchard, where in 1996 and 1997 pistachio trees near the almond orchard had substantially higher NOW infestation. The results suggest that sanitation by removing the mummy nuts should reduce NOW infestation levels and *Aspergillus* and aflatoxin contamination.

Figs. Although first-crop Conadria figs from the 1996 harvest had substantially more fungal decay than main-crop figs, first-crop figs did not consistently have more decay caused by fungi in *Aspergillus* Sect. *Flavi*. During the 1997 harvest, samples of first-crop figs were collected from three Conadria orchards and one Calimyrna orchard and are in the process of being evaluated.

In the final year of the irrigation experiment, the incidence of figs colonized by *Aspergillus* Sect. *Flavi*, the incidence of figs with BGY fluorescence, and level of aflatoxin decreased

with increasing amounts of applied water in summer. Therefore, fig growers should avoid imposing drought stress in their orchards. Preparations have been made for another irrigation experiment, which will investigate the effect of burying drip lines on *Aspergillus* decay of figs.

Studies on the association of bright greenish yellow fluorescence (BGYF) to aflatoxin contamination in California figs were completed. BGYF in 265 naturally infected figs (for which the fungus was also isolated and identified) was associated with decay by only four fungal species: *A. flavus* (both L and S strains), *A. parasiticus*, *A. tamarii*, and *A. alliaceus*. This is the first report that infections of *A. alliaceus* are associated with BGYF. For the 1996 harvest, infected figs that had external blemishes (more likely to be removed during processing) were more likely to show BGYF externally (25% of such figs) than figs with no external blemishes (none showed external BGYF). In addition, infected figs showing no external blemish were more likely to show no BGYF (59% of these figs) than figs with blemishes (only 13%). This lack of BGYF in some figs infected with *Aspergillus* Sect. *Flavi* is probably not due to the fungal strain or isolate involved, because six isolates previously obtained from nonfluorescent figs did produce BGYF in figs inoculated in 1997. The size of the fungal colony, however, is related to BGYF, because only 53% of the naturally infected figs (from 1996 harvests) that had colonies smaller than 10 mm diameter showed BGYF, while 91% of the figs with larger colonies showed BGYF. Aflatoxin analysis of inoculated wounded figs at various developmental stages were completed. In results similar to the previous year, wounding substantially increased aflatoxin production for green figs (164 and 13,892 ppb total aflatoxins for nonwounded and wounded, respectively) but not for mature brown figs (7,007 and 9,549 ppb). These results suggest that wounding figs during harvesting would not increase aflatoxin contamination.

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DETECTION, SORTING, AND TESTING OF AFLATOXIN CONTAMINATED PISTACHIOS AND ALMONDS

T.F. Schatzki, T.C. Pearson, L. Le, R. Miller, M. Ong, USDA, ARS, WRRC, Albany, CA

An algorithm is being developed for automated recognition of insect infested almonds by x-ray imaging. High resolution film x-ray images were used for the algorithm development after low pass filtering to obtain a comparable resolution to x-ray line scan images. Insect tunnels are highlighted by first removing background noise by image clipping, then performing a local (5x5) histogram equalization, then a global histogram equalization. This enhanced imaged is converted to a binary image by thresholding, then one 3x3 erosion followed by six sequential 3x3 dilations isolate the insect tunnels. Glob identification then determines if a nut contains an insect tunnel. A sample of 518 insect infested almonds was found to contain 619 insect tunnels by manual inspection of film x-ray images. From this set of 619 insect tunnels, 520 (84%) were identified by the computer vision algorithm. From a set of 511 normal almonds, 89 (17%) were falsely identified as having an insect tunnel by the algorithm.

Testing of a previously reported real time image sorter (Pearson. T., Lebensm. u. Technol., 1996, 28(6):203-209) for stained pistachio nuts was performed at a California pistachio processing plant. Sorting rates matched commercial speeds of 140 kg/hr. Tests were performed on three process streams: second pass color sorter reject nuts, size 21/25 color sorter accept nuts, and hand sort reject nuts. The image sort reject nuts of the color sort accept stream, comprising 2% of the sorted nuts, were found to contain 3.7 ppb aflatoxin while the image sort accepts contained 0.0% aflatoxin. Test results of the color sort accept stream indicate that the image sorter is removing most of the aflatoxin contaminated nuts, presumably by detection of early split staining patterns, which were not rejected by the color sorters.

An aflatoxin testing protocol has been proposed to significantly reduce the quantity of nuts required to be analyzed while maintaining a high confidence of the mean aflatoxin measured. The protocol involves sorting a large sample of pistachio nuts with the previously mentioned image sorter and only analyzing the reject, or stained, portion. The images sorter tests on the color sort accept stream indicate that the image sorter can isolate all of the aflatoxin in ready for sale product into a subset comprising less than 2%. By analyzing only the rejects of the image sorter, the sample size required for analysis to achieve 4±2 ppb aflatoxin is reduced from 355 kg to 7 kg.

Session 1

POSTER PRESENTATIONS

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SEGMENTATION AND CLASSIFICATION FOR X-RAY AGRICULTURAL PRODUCTION INSPECTION

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Processing of real-time X-ray images of randomly-oriented and touching pistachio nuts for product inspection is considered. We describe the image processing used to isolate individual nuts (segmentation). This involves a new watershed transform algorithm. Segmentation results on approximately 3000 X-ray (film) and real time X-ray (linescan) nut images were excellent (>99.9% correct). Initial classification results are also presented that indicate that the percentage of infested nuts can be reduced to 1.6% of the crop with only 2% of the good nuts rejected; this performance is much better than present manual methods and other automated classifiers have achieved.

Key Words: Classification, detection, morphological processing, product inspection, watershed transform, segmentation, X-ray sensors.

ADVANCES IN INSECT-ORIENTED METHODS FOR REDUCTION OF MYCOTOXINS IN CORN - FY 1997

P. F. Dowd[1], R. J. Bartelt[1], R. W. Behle[1], M. R. McGuire[1], R. A. Norton[1], R. L. Pingle[1], B. S. Shasha[1], I. Haas[2], J. A. Duvick[3], D. L. Dornbos[4], D. F. Kendra[4], T. A. Green[5], D. R. Penland[6], E. J. Faron[6], R. S. Boston[7], A. Mehta[7], and L. M. Lagrimini[8]
[1]USDA, REE, ARS, NCAUR, Peoria, IL; [2]Brigestone, Morton, IL; [3]Pioneer Hi-Bred, Int., Johnston, IA; [4]Novartis Seeds, Stanton MN; [5]Gempler's, Mt. Horeb, WI; [6]Cerestar U.S.A., Inc., Hammond, IN; [7]Dept. Botany, N.C. State, Raleigh, NC; [8]Dept. Horticulture and Life Sciences, Ohio State U., Columbus, OH.

Plant Resistance

(Poster Presentation - Activity of a Novel Corn Protein Against Insects by P. F. Dowd, A. Mehta and R. S. Boston). A corn protein concentrated in the endosperm was tested against several corn feeding insects and a few other insects at a concentration (1000 ppm) within the natural range in the kernel. The protein was variable in activity. The pro-form had no significant affect, but the enzyme-activated form killed 71% of cabbage loopers, but had no affect on Indian meal moths. Activity ranged (in order of increasing activity: Indian meal moth < fall armyworm < corn earworm, European corn borer < cabbage looper. In choice assays, both the proform and the enzyme-activated form were approximately equally active against maize weevils, Freeman sap beetles, dusky sap beetles and strawberry sap beetles. For the beetles, feeding was reduced by from 33% (Freeman sap beetle) to 6-fold (dusky sap beetle). There is thus evidence that the sensitivity of caterpillars is related to the stage of corn (if corn feeders), but less so for the beetles. Preliminary analysis suggests the maize weevils (only beetle tested so far) may be capable of digestively activating the protein.

Detailed assays of kernels from commercial hybrid corn ears damaged by insects (primarily European corn borers) indicated that fumonisin concentrations, like aflatoxin concentrations, are much higher in insect-damaged kernels. However, the degree of visible mold and age (as indicated by pericarp color change) the kernel was damaged influenced levels. In general, insect-damaged kernels with discolored pericarps (often with visible mold) comprise from 1-10% of the weight of kernels from the ears, but contain from 30-95+% of fumonisins from the ears. Insect damaged kernels often had a few to several hundred ppm of fumonisins, and some had over 1000 ppm. Damaged kernels without pericarp discoloration rarely had more than a few ppm of fumonisin. Thus eliminating insect damage can significantly reduce not only aflatoxin (as has been reported before) but also fumonisin.

Studies with commercial Bt hybrid corn were expanded from 1996 to include comparisons with commonly planted Pioneer Hi-Bred, Northrup King, and CIBA (now both Novartis Seeds) lines. The Northrup King and Pioneer Hi-Bred lines (which have high levels of the Bt protein throughout the plant) were virtually free of caterpillar (primarily European corn

borer) damage, although some minor damage associated with mold by corn earworms and sap beetles did occur. Corn borer damage in nonBt versions of these hybrids sometimes was above 90%, with high incidence of molded kernels as well. Results with the CIBA Bt line (which only expresses low levels in kernels and silks) were similar to those reported last year. Although corn borer incidence was similar, the numbers of early damaged kernels (with discolored pericarps) of the Bt hybrid were ca. 3-fold less, and associated *Fusarium* molded kernels, ca. 4-fold less per ear. No visibly sporulating *A. flavus* was noted. Mycotoxin analysis of kernels from this study are pending.

Leaves of tomato plants expressing high levels of tobacco anionic peroxidase were fed on less by first instar corn earworm larvae, and had smaller feeding holes when damaged, compared to wild type plants. As part of a USDA-ARS, Pioneer Hi-Bred CRADA, corn peroxidase roles in disease resistance are continuing to be explored.

Insect Monitoring and Control

Adherent corn flour encapsulated malathion (1% active ingredient) was formulated to a granule size approximating that of commercial formulations of Lorsban (15% active ingredient). Both were applied by air to six, 1-acre plots (9-lb malathion granules/A and 6 lb of Lorsban granules/A) of speciality corn when numbers of European corn borers exceeded treatment thresholds. Coverage by the treatments appeared to correspond to application rates. At 2 weeks after application, both the malathion and Lorsban has significantly reduced the incidence of kernel damage by caterpillars (mostly European corn borers), with the malathion being more effective in reducing the total amount of caterpillar damage per ear. The malthion reduced the % of ears with >10 kernels damaged per ear by 200% (vs. 20% for the Lorsban). The malthion treatment also significantly reduced the numbers of sap beetles present. No significant affect on predator populations was noted for either treatment in this case, although predator levels were very low overall, even in control plots.

Autoinoculative release of the insect pathogen *Beauveria bassiana* to control sap beetles in mass overwintering sites was continued. Earlier introduction of the autoinculator, prior to harvest, appears to have helped dispersal of the material, based on the greatly increased incidence of *B. bassiana* recovered from beetles in the spring compared to prior years. Strain confirmation is pending. A relatively high incidence of *A. flavus* was recovered from sap beetles collected from specific trap locations in the late spring. This presence parallels the postulated buildup in 1988 during the dry but seasonable spring. Lack of visible *A. flavus* in corn in 1997 at the research site confirms the biphasic predictive model for aflatoxin in the corn belt under development, which indicates conditions for buildup must be present prior to stressing of corn. High temperatures/limited rainfall occurred in 1995 and 1996 at this site during corn fill, but springs were wet, preventing *A. flavus* buildup. At this site in 1997, temperature and rainfall during kernel fill were relatively normal (and at some points cooler than normal), so built up *A. flavus* apparently could not readily establish in stressed corn. This information should allow for the completion of the computer model. Modification in the trap/autoinoculator design have allowed for more

simplified manufacture, such that manufacture and distribution of the trap/autoinoculator (patented by ARS) is more probable.

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Poinar, G. O., Jr. and Dowd, P .F. 1997. *Psammomermis nitidulensis* n. sp. (Nematoda: Mermithidae) from sap beetles (Coleoptera: Nitidulidae) with biological observations and a key to the species of *Psammomermis*. Fund. Appl. Nematol. 20: 207-211.

AFLATOXIN ACCUMULATION IN TRANSGENIC BT CORN HYBRIDS AFTER INSECT INFESTATION

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Infection of corn, *Zea mays L.*, with *Aspergillus flavus* and subsequent accumulation of aflatoxin in the grain is a sporadic problem in the Corn Belt and a chronic problem in the South. A potent carcinogen, aflatoxin affects humans and livestock. Drought, high temperatures, and insect damage have long been associated with increased aflatoxin contamination. Injuries to ears of corn caused by insect feeding provide sites for fungal infection. Resistance to insect feeding should, therefore, be an effective method for reducing fungal infection and aflatoxin accumulation in the grain. Development of transgenic corn plants expressing insecticidal proteins isolated from the bacterium *Bacillus thuringiensis* could prove useful in reducing insect damage and subsequent fungal infection. In this investigation commercially available transgenic and nontransgenic corn hybrids were infested with fall armyworm (*Spodoptera frugiperda*), southwestern corn borer (*Diatraea grandiosella*), and corn earworm (*Helicoverpa zea*)larvae and simultaneously inoculated with an *A. flavus* spore suspension 7 days after midsilk to determine the effectiveness of transgenic corn hybrids in reducing damage by the three insects and associated *A. flavus* infection and aflatoxin accumulation. Ears were harvested 56 days later and visually evaluated for the presence of insect damage and *A. flavus* growth. Aflatoxin contamination was determined using the Vicam Aflatest (Watertown, MA). The highest levels of damage resulted from the southwestern corn borer feeding. In the transgenic hybrids with expression of the insecticidal proteins in kernel tissues (N7590B*t* and N7639B*t*), southwestern corn borer damage was significantly reduced. Damage from fall armyworm and corn earworm was not affected. When inoculated with an *A. flavus* spore suspension visible fungal growth was present on ears of both the transgenic and nontransgenic hybrids. The CryIA(b) protein in these hybrids did not prove to be effective in reducing aflatoxin contamination in the grain either directly or indirectly as a consequence of reduced insect feeding.

AFLATOXIN CONTAMINATION OF COMMERCIALLY GROWN TRANSGENIC BT COTTONSEED

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Transgenic Bt cotton may have reduced susceptibility to aflatoxin contamination as a result of pink bollworm resistance. During 1995 and 1996, Bt cottonseed from several commercial fields in Arizona contained aflatoxin levels unacceptable for dairy use. Comparison of cottonseed with and without BGYF (bright-green-yellow fluorescence) from one highly contaminated (>6,000 ppb aflatoxin B_1) Bt seed lot indicated that most of the observed contamination probably was formed during the second phase of contamination when the mature seed cotton was exposed to high humidity. Seed exhibiting BGYF was repeatedly detected in Bt cottonseed lots but, pink bollworm exit holes were not observed in the field. Pink bollworm entrance holes were not found to predispose bolls to contamination. A field plot test in 1996 demonstrated high resistance among Bt cultivars to both pink bollworm damage and formation of BGYF seed cotton. These observations suggest that resistance to pink bollworm will result in reduced aflatoxin contamination when pink bollworm pressure coincides with conditions conducive to *Aspergillus flavus* infection. However, Bt cultivars do not have innate resistance to *A. flavus* infection and are not resistant to aflatoxin increases occurring after boll opening. Large quantities of aflatoxins can form during this second phase of contamination. Therefore, if the insect protection provided by Bt cotton means growers will hold the crop in the field longer, the benefits of Bt cotton to management of aflatoxin contamination may be lost.

INFLUENCE OF HARVEST DATE ON AFLATOXIN CONTAMINATION IN WESTERN ARIZONA IN 1995 AND 1996

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Aspergillus flavus infection of cottonseed can result in aflatoxin contamination. If aflatoxin content exceeds 20 parts per billion (ppb) regulation prohibits its use as a dairy feed. If aflatoxin content exceeds 300 ppb then the cottonseed cannot be used as beef cattle feed. In both 1995 and 1996 a gin in western Arizona separated cottonseed at ginning by field of origin. Seed lots were kept distinct until after aflatoxin analyses had been performed by a commercial laboratory. Regression analysis showed that late harvest resulted in greater aflatoxin content in both years. The proportion of seed lots discounted for aflatoxin contamination in excess of 20 ppb was 89% in 1995 and 79% in 1996, and those discounted for contamination in excess of 300 ppb was 46% and 30% of seed lots in 1995 and 1996 respectively. In 1995 no crop harvested after JD 252 had less than 20 ppb, and after JD 301 no crop had less than 300 ppb. In 1996 no crop harvested after JD 253 had less than 20 ppb, and after JD 303 no crop had less than 300 ppb. In Arizona, the risk of unacceptable aflatoxin contamination can be reduced by early harvest.

ASSESSMENT OF RISK OF MYCOTOXIC DEGRADATION OF STORED MAIZE IN NIGERIA AND BENIN REPUBLIC, WEST AFRICA

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An inventory of maize grain quality in West African peasant farming systems was conducted from 1993 to 1995. The objectives were: 1)to determine crop husbandry, harvest, and storage practices that rendered stored maize more vulnerable to aflatoxin; and 2) to determine if there were differences in risk of contamination among the different agroecological zones of West Africa. Interviews were conducted with 425 small-scale farmers in Nigeria and Benin and samples were taken one, three, or six months after storage of the maize. Flora and fauna were recorded, and semi-quantitative detection of aflatoxin was conducted using TLC. Principal components and regression analyses were used to determine which factors were significantly related to aflatoxin risk.
In Nigeria, around 25% of the stores were aflatoxin positive with an average of 292 ppb. In Benin, from 27-73% of the stores were contaminated with an average of 37 ppb aflatoxin during the 2 year period. Maize in all zones was found to have risk of contamination, but the degree of risk by zone was not consistent between countries, indicating that farming practices had as much influence as climate. The Humid Forest zone had significant risk only in Nigeria when farmers stored maize on the floor of a room. The Southern Guinea Savanna had high risk in both countries, with 25-30% of the stores contaminated early in the storage period. Factors significantly related to high toxin levels in that zone were insect damage, crop system, prolonged field drying, and sorting practices. Maize stores in the Northern Guinea savanna of Nigeria had the lowest incidence and the least toxin contamination, while in Benin the highest incidence,73%, occurred in this zone in 1994. Crop rotation, use of insecticide and fertilizer, field drying, and storage system were significant factors related to contamination. The Sudan Savanna, bordering the Sahel, had the most consistent risk of high aflatoxin contamination, averaging 305 ppb across years. In summary, maize is a primary staple for human consumption in Benin and Nigeria, and many people are being exposed to aflatoxin levels well above accepted standards. Little is known about the year to year variability of contamination in West Africa so it is unclear if the contamination levels during this sampling period were typical.

DIFFERENCES IN THE METABOLIC RESPONSE OF PEANUT PLANTS TO WATERS STRESS

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In peanut, drought stress enhances *Aspergillus flavus* invasion and aflatoxin contamination. Decreased phytoalexin production and altered metabolite levels under water stress conditions is believed to favor aflatoxin production in peanut. The objective of this study is to determine the differences in the metabolic response of young and mature peanut plants, and between drought tolerant and susceptible lines to water stress.

Seed of drought susceptible (Florunner) and drought tolerant (108-F) peanut lines were obtained from C.C. Holbrook, USDA/ARS, Tifton, GA, and grown in the greenhouse at Florida A&M University. Water stress was imposed for 2 to 23 days by withholding water at two developmental stages: one at 32 days after planting and another at 108 days after planting. Leaf samples were collected from these plants, freeze-dried and analyzed for soluble and insoluble carbohydrates, free amino acids and proteins.

The results showed that in both young (32 day old) and old (108 day old) peanut plants, free amino acids, soluble carbohydrates and protein content increased while the insoluble carbohydrates content decreased following their exposure to water stress. However, in drought susceptible line (cv.Florunner) free amino acids, soluble carbohydrates and protein levels increased up to 18 days of water stress and then decreased. In contrast, in drought tolerant line (108-F), free amino acids, soluble carbohydrates and protein levels increased up to 23 days of water stress.

These data indicated that response of young and mature peanut plants to water stress was similar, and that the drought tolerant peanut lines accumulated stress metabolites for a longer period than the drought susceptible line. This would suggest that young peanut plants could be used in screening studies for selecting drought tolerant lines. In addition, peanut lines capable of accumulating stress metabolites for a longer period under water stress conditions are able to withstand drought stress better and thus may have a lower incidence of aflatoxin contamination.

POTENTIAL USE OF NATURAL PRODUCTS FOR PREVENTION OF FUNGAL INVASION AND/OR AFLATOXIN BIOSYNTHESIS IN CROPS

PANEL DISCUSSION

PANEL DISCUSSION TITLE: Potential Use of Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Biosynthesis in Crops

PANEL MEMBERS: Deepak Bhatnagar (Chair), Bruce Campbell, Robert A. Norton, Harold Gardner, John Linz and Biing-H. Liu

SUMMARY OF PRESENTATIONS: A detailed understanding of the molecular regulatory processes that govern aflatoxin biosynthesis has lead to a more rapid identification and characterization of natural products that could prevent fungal invasion and/or aflatoxin production. Salient features of the presentations at this workshop are as follows:

(1) Several plant metabolites and proteins have been identified that could potentially reduce fungal infection and aflatoxigensis.

(a) Naphthoquinones in walnut hulls inhibit germination of *A. flavus* spores.

(b) A combination of host plant volatiles and sexual pheromones improve trap capture of major insect pests that create wounds allowing for fungal infection.

(c) Secondary products of the lipoxygenase pathway e.g., 13-hydroperoxylinoleic and 9-hydroperoxylinoleic acid suppressed growth of aflatoxigenic fungi and inhibited aflatoxin production.

(d) The phenolic signal molecules, acetosyringone, syringaldehyde and sinapinic acid, were found to inhibit aflatoxin production by *A. flavus*.

(e) β-carotene was found inhibit aflatoxin production by several *A. flavus* genotypes studied.

(f) Production of fermentable sugars in corn kernels during breakdown of kernel starch by fungal alpha amylase may induce aflatoxin biosynthesis.

(g) Volatile aldehydes from aflatoxin-resistant varieties of corn inhibit fungal growth.

(h) Two inhibitory activities have been identified in corn inbred shown to be resistant to fungal invasion, one activity inhibits aflatoxin promotion and the other one inhibits fungal growth. The activity inhibiting fungal growth is associated with a chitmase.

(i) A 14kDa trypsin inhibitor was found to be associated with resistance to *Aspergillus flavus* infection in several genotypes.

(j) It was determined that both storage proteins and storage carbohydrates in oilseeds influence the vulnerability of oilseed crops to aflatoxin contamination.

(2) Significant progress has been made on characterization of the biological mechanisms that govern gene and protein functions specific to aflatoxin formation. These studies have been included the following:

(a) It was demonstrated that although aflR is the regulatory gene that is essential for aflatoxin production, the AFLR protein alone is not sufficient to induce aflatoxin production.

(b) The expression of aflJ appears to enhance the functionality of aflR.

(c) An AFLR-repressor interaction may be involved in the nitrate inhibition of aflatoxin biosynthesis in *A. parasiticus*. A threshold of aflR gene product was required for the alleviation of nitrate inhibition of aflatoxin synthesis. This phenomenon may not occur during sterigmatocystin production in *A. nidulans*, where no such nitrate inhibition is observed. An unusual *A. flavus* strain was isolated in which, unlike other *A. flavus* strains, ammonium inhibited aflatoxin production but nitrate stimulated toxin formation.

(d) Site-directed mutagenesis of aflR and ordA genes allowed for the identification of the regions of the DNA that were vital to the production of a functional protein (AFLR) required for regulation of aflatoxin pathway, or for catalysis of a step of the pathway (ORDA).

(e) A comparison of various genes in the gene cluster of sterigmatocystin-producing *A. nidulans* and aflatoxigenic *A. flavus/parasiticus* will provide significant information into the uniqueness of *A. flavus* and *A. parasiticus* in their ability to infect crops and produce aflatoxins. A study of the regulatory gene (aflR) from various *Aspergillus* species suggests that although the AFLR protein is produced by several Aspergilli, only the protein produced by aflatoxigenic fungi has the ability to activate pathway genes.

(f) Indirect immunofluorescence microscopy has been used to localize gene products essential for aflatoxin biosynthesis. Varying degrees of protein-products of several genes were identified in spores, conidia, and sclerotia. It may be possible that aflatoxins are concentrated in specific fungal structures which are important for fungal survival and colonization.

SUMMARY OF PANEL DISCUSSION: The discussion was initiated by a question to Bob Norton as to why the effects of sucrose and corn oil on aflatoxin production were not additive, especially when these individually enhanced aflatoxin production and collectively inhibited it. Bob replied that it was possibly because of the way one nutrient source was metabolized in the presence of the other, as compared to when they were present as the sole source. It was suggested to Bruce Campbell to develop a mixture of walnut, pistachio and other treenut hulls because each of these produce compounds that affected fungal growth or development and/or aflatoxin production. Bruce was also asked if he knew where the phenolic lipids from trees inhibited the aflatoxin pathway. Bruce said they were working on assessing the mode of action of the lipids. Hal Gardner was asked if he knew the conditions under which the lipoxygenases were functional in soybeans; to which he responded that it was unknown. Biing Liu was asked why the *A. nomius* AFLR protein was larger than the 47kDa protein from

other *Aspergilli*, and if adding salt to her purification column would have an effect on the protein size by breaking up any protein-protein interactions. She did not think that the *A. nomius* protein size was due to protein-protein interactions.

John Linz was asked if *aflR* was sufficient for regulating aflatoxin biosynthesis. To which he replied that there is increasing evidence from his lab and that of others that suggests that AFLR protein alone is not sufficient to activate toxin synthesis. There could be repressors (Bhatnagar's and Linz's labs) or activators (such as *aflJ*, Payne's lab). Linz highlighted his observation that the aflatoxin pathway proteins were localized in various fungal structures and Biing Liu added that the AFLR is present in cytoplasm.

The major part of the discussion centered around the nitrate effect on inhibition of toxin synthesis mentioned by Deepak Bhatnagar. Peter Cotty argued that the nitrate is utilized by the fungus as a nitrogen source and aflatoxin was produced under these conditions. Gary Payne replied that this was true only when ammonium was present with nitrate. But when nitrate alone was present, higher levels of nitrate inhibit toxin synthesis by inhibiting the aflR gene function (Bhatnagar's lab). He emphasized that this effect had been observed by many scientists, including Payne and Bhatnagar. And Bhatnagar emphasized that from his work it was clear that the nitrate effect was opposite in *A. nidulans*. Also, since Cotty had identified an unusual aflatoxin producing strain of *A. flavus* that was inhibited by ammonium and stimulated by nitrate (much like *A. nidulans*), this strain would be very valuable in studying the nitrate phenomenon in regulating toxin synthesis. Peter felt that the nitrate effect may be related to altered pH, and Nancy Keller has identified some pH effects on aflatoxin biosynthesis. However, Bhatnagar and Payne felt that the effects of nitrate is not solely due to pH shift. Further, Bhatnagar's lab has clearly demonstrated that there may be protein: aflR interactions in response to nitrate, preventing toxin synthesis.

PLATFORM PRESENTATIONS

ROLE OF NATURAL PRODUCTS, SEMIOCHEMICALS AND MICROBIAL AGENTS IN REDUCING INSECT INFESTATIONS, *ASPERGILLUS* INFECTION AND AFLATOXIGENESIS IN TREE NUTS

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Aflatoxin contamination of tree nuts is a domestic food-safety and international trade issue. The US tree nut (almonds, walnuts and pistachios) crop for 1996 was valued at > $1.4 billion, almonds alone > $1 billion. Over 70% of these tree nuts are exported. Major importing nations (EU and Asia) set strict tolerance thresholds (< 20 ppb) for aflatoxin. Reducing aflatoxin contamination and infection by aflatoxigenic aspergilli in tree nuts are important to overall exportability of tree nuts, in addition to providing a safe product for domestic consumption.

There are two main efforts underway in the Plant Protection Research Unit to reduce or eliminate tree nut contamination by aflatoxins; control of major insect pests and control of either *A. flavus* or its ability to synthesize aflatoxins. Both efforts entail environmentally benign approaches, wherein, biocompetitive microorganisms or natural chemical constituents of host-plants, insects, or fungi are used to deter insect attack, fungal growth or aflatoxin biosynthesis.

Feeding by larvae of major insect pests creates wounds to tree nut kernels allowing infection by aflatoxigenic aspergilli. Combining host plant volatiles (HPVs) with sexual pheromones improves trap captures of adults (moths) of these pests, in some instances. Tested HPVs are effective during early and post-harvest seasons. Lack of effect during nut maturation and hull split indicates 'odor phenology' may play a role. Examination of HPVs of leaves, hull-split and alternate host fruits indicate vast differences and changes in 'volatile profiles'. Effect of volatile profile changes on HPV-synergism of pheromones will be examined.

A number of findings were made concerning control of aflatoxigenic aspergilli in tree nuts. Naphthoquinones in walnut hulls inhibit germination of *A. flavus* spores. Anacardic acids in pistachio hulls inhibit aflatoxin biosynthesis. Rehydration of post-processed, close-shelled pistachios can become endogenously contaminated with aflatoxin. Almond lines exhibit inherent differences in supporting aflatoxin biosynthesis suggesting a genetic component. A number of indigenous yeasts were isolated that inhibit growth of *A. flavus*.

INTERACTION BETWEEN SUGARS, SUGAR METABOLITES AND TRIGLYCERIDES IN THE PRODUCTION OF AFLATOXIN BY *A. FLAVUS* NRRL 3357

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The objective of this work was to determine what effect combinations of sucrose and triglycerides would have on aflatoxin B_1 (AFB_1) production by *Aspergillus flavus* NRRL 3357. Several studies have indicated that the germ of corn is the principle site of aflatoxin synthesis. The main nutrients in germ are triglycerides (TGs), which account for ca. 35 wt% of the germ, and sucrose which can go as high as 7%. To assess the relative contribution of these two nutrients to AFB_1 production I tested refined corn oil, TGs composed of single fatty acids, sucrose and selected glycolysis and TG metabolites separately and as binary mixtures. The suspended disc culture method (Mycopathologia 1995, 129:103) was used for all work.

Interaction between sucrose and triglycerides was addressed with an experiment in which levels of sucrose (0, 0.5, 1, 2, 4, and 8%) were tested against levels of corn oil (CO) of 0, 0.5, 1, 2, 4, 8 and 16%. AFB_1 production increased as the level of sucrose in the medium increased from 0.5% to 8% when sucrose was the sole carbon source. Toxin production on CO alone increased at levels up to 16% and decreased by 16% at 32% oil. Production of AFB_1 on CO was over two times that on the same percentage of sucrose. Mixtures of CO and sucrose showed that sucrose significantly decreased utilization of CO for aflatoxin formation with a maximum decrease in toxin production at 2% sucrose versus CO levels of 4-16%. Above 2% the contribution to AFB_1 production from sucrose began to offset the inhibition of CO utilization but never reached the level of CO alone. Aflatoxin production on 16% CO was 296 ug/mL but when 2% sucrose was present the level was 136 µg/mL - a 60% reduction, although growth increased from 18 to 20 mg/mL. When sodium oleate was used as a carbon source no inhibition occurred with 5% glucose. A defined mixture of TGs with a composition similar to CO, composed of 31% trioleate, 56% trilinoleate and 13% tripalmitin, gave results similar to those obtained when CO was used as a sole carbon source.

It is known that there are significant differences in the ability of various sugars to support aflatoxin synthesis. However, how different sugars might affect growth and AFB_1 formation on oil-containing medium has not been systematically investigated, e.g. do sugars and unphosphorylated glycolysis and TG metabolites affect CO utilization for AFB_1 in the same way? Growth and AFB_1 was compared for seven sugars, three metabolites and CO; separately and together at 2% sugar/metabolite and 10% CO. Four general patterns of interaction were found, exemplified by results for glucose, glycerol, glyceraldehyde (GA) and dihydroxyacetone (DHA): glucose produced high levels of toxin when used alone but inhibited TG utilization for toxin, but not growth. Glycerol gave high levels of AFB_1 alone and was additive with CO for AFB_1 production. DHA allowed growth but almost completely inhibited AFB_1 alone and over 93% with TG.

Glyceraldehyde had virtually no toxin production alone but stimulated toxin production ca. 35% with TG. The appearance of the fungus differed markedly for each of these compounds as well. A consistent model for how these results fit together is not obvious but they show that the production of aflatoxin from triglycerides is markedly affected by the type of sugar available to the fungus.

α-Linolenic acid is a very minor component of corn oil (usually <1%) however others have noted that it can sharply inhibit *A. flavus* spore germination, a result that was confirmed here with tri-γ-linolenate (tri18:3). Mixtures of trioleate (tri18:1) and tri18:3 suggested that the level present in germ could have a significant effect on AFB_1 and therefore that linolenic acid level might affect aflatoxin production. Mixtures of tri18:1 and tri18:3 (%/% of medium: 7/3, 8/2, 9/1, 9.5/0.5, 9.75/0.25, 9.9/0.1) resulted in increases of 34% in AFB_1 at 9.75%/0.25% but toxin decreased to nearly zero at a ratio of 8%/2%. Germ, however, has high levels of antioxidant tocopherols, principally α- and γ-tocopherol. These were tested for their ability to offset inhibition by tri18:3. There was no difference in effect for (±)-α-, (+)-α-, and (+)-γ-tocopherol when tri18:3 was used as a carbon source (see below) and (±)-α-tocopherol (α-tocol) was used subsequently. The addition of 100 μg/mL of α-tocol eliminated both growth and aflatoxin inhibition and levels as low as 5 μg/mL increased AFB_1. α-Tocol increased AFB_1 production by 99.8% with tri18:3, by 12% with tri18:2 and decreased AFB_1 by 24% with tri18:1 when added at 0.25 mg/mL, a typical level in corn germ. It also increased production from refined CO. Aflatoxin level on 5% TG medium with α-tocol was 169, 164, and 142 μg/mL for tri18:1, tri:18:2 and tri18:3 respectively. The comparable figures for medium containing 5% sucrose were 118, 126 and 154 μgm/mL. In each case tri18:3 was significantly different.

In summary it is clear that the complex nutrient milieu of the germ; consisting of various sugars and triglycerides, may affect aflatoxin metabolism in unexpected ways - e.g. the effect of higher sucrose levels and higher TG levels is not additive. Certain sugars appear to prevent triglyceride utilization for aflatoxin synthesis while others do not. Tocopherols at certain levels prevent fatty acid oxidation products from inhibiting aflatoxin formation and their presence in a medium utilizing polyunsaturated fatty acids is strongly suggested.

SOYBEAN LIPOXYGENASE IS ACTIVE ON NONAQUEOUS MEDIA AT LOW MOISTURE: A CONSTRAINT TO XEROPHILIC FUNGAL GROWTH?

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Many studies have implicated secondary products of the lipoxygenase pathway as inhibitors of *Aspergillus* growth and aflatoxin production. More recently, Burow et al. (Molec. Plant-Microbe Interactions 10: 380-387 [1997]) have shown that the primary product of soybean lipoxygenase-1, 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid, suppressed growth of *Aspergillus* species and inhibited aflatoxin production at the gene level by suppressing the expression of a ketoreductase in the aflatoxin biosynthetic pathway. However, *Aspergillus* can grow on "dry" crop seeds equilibrated at relative humidities at about 85% or higher implying that lipoxygenase activity may not occur in dry media. Since previous work has not convincingly demonstrated that lipoxygenase can catalyze fatty acid oxidation at low humidities, research was designed to answer this question. Soybean extracts (pH 7.5) and linoleate (pH 7.5) were mixed separately with cellulose, and then dried overnight by vacuum desiccation. It was determined that the cellulose composite required 7 hours to achieve moisture equilibrium at the designated relative humidities, thus experiments were completed after an additional overnight equilibration at the designated relative humidity before mixing the cellulose/linoleate with cellulose/soybean extract. Four relative humidities ranging between 52% and 95% were tested. Controls were treated the same, except the soybean extracts were inactivated by heating prior to mixing with cellulose. Oxidation of linoleate occurred at all relative humidities compared to much lower values of the controls. There was a notable positive correlation in oxidation rate with increasing relative humidity. That the oxidation was principally enzymatic was shown by chiral analysis of the linoleate hydroperoxides formed. The main product was 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid followed by significant percentage of 9*S*-hydroperoxy-10*E*,12*Z*-octadeca-dienoic acid. Because the "dry" reaction products were somewhat more racemic than a short-time aqueous incubation of soybean extract with linoleate at pH 7.5, autoxidation appeared to be initiated by the lipoxygenase reaction in dry media. Experiments concerning the effect of our test conditions on *Aspergillus* growth and aflatoxin production are currently underway.

THE EFFECTS OF GROWTH CONDITIONS AND DEVELOPMENT ON AFLATOXIN SYNTHESIS

J. Linz, Michigan State University, E. Lansing, MI

For details, refer to the following abstracts:

"Molecular characterization of the effects of nutritional factors on transcription and aflatoxin production by reporter strains of *Aspergillus parasiticus*" (M. Rarick, M. Miller and J. Linz) (page 85)

"The Immunodetection of gene products involved in aflatoxin biosynthesis in *Aspergillus parasiticus*" (L.-W.Lee, C.-H. Chiou and J. E. Linz) (page 86)

GENE EXPRESSION SYSTEMS IN AFLATOXIGENIC FUNGI FOR MONITORING AFLATOXIN ELABORATION IN CROPS

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Over the last few years significant information has been obtained with respect to HOW aflatoxins are produced by the fungus. The gene cluster affecting the aflatoxin biosynthetic pathway has been identified and characterized, and a significant understanding of the molecular regulation of the pathway has been established. Additional details on the genetics of the pathway are still being worked out. Recently, a gene for a dehydrogenase (*adhA*) and a cytochrome P-450 coding gene (*avnA*) have been identified and characterized. Further, a gene, *aflJ*, has been identified in Dr. Gary Payne's lab, and our lab is working with him to identify the exact role of this gene. One thing that is certain is that this gene is involved in the regulation of the aflatoxin pathway along with the regulatory gene, *aflR*.

With these recent developments in the genetics of toxin synthesis, and the observation from our lab and those of others (J. Linz and N. Keller) that toxin synthesis and fungal development may have a common regulator, the WHY of aflatoxin biosynthesis may be better understood. In that process, the genetic similarities and differences between the toxigenic fungi *Aspergillus flavus*, *A. parasiticus*, and *A. nidulans* will be very useful. A few subtle and some significant differences exist between these fungi; not as much between *A. flavus* and *A. parasiticus*, but more between these two fungi and *A. nidulans*:

(a) *A. flavus* and *A. parasiticus* are distant cousins of *A. nidulans* morphologically.

(b) *A. flavus* and *A. parasiticus* produce aflatoxins (*A. flavus* only B_1 and B_2, whereas *A. parasiticus*, B_1, B_2, G_1 and G_2), whereas *A. nidulans* produces no aflatoxin but produces the penultimate aflatoxin pathway intermediate, sterigmatocystin (1).

(c) The temperature requirement for optimal toxin production varies in these fungi: 37°C for sterigmatocystin in *A. nidulans*, and 30°C for aflatoxin production in *A. flavus* and *A. parasiticus* (1, 2), suggesting metabolic differences.

(d) *A. nidulans* has a sexual stage, but *A. flavus* and *A. parasiticus* propagate only asexually.

(e) The organization of the aflatoxin pathway gene cluster in *A. flavus* and *A. parasiticus* is more or less identical, but the organization of the same genes in *A. nidulans* genome is different (Figure 1). Also, the intergenic distances are very small in *A. nidulans* but more prominent in *A. flavus* and *A. parasiticus* (3, 4). These differences may have some evolutionary significance.

(f) It appears that *aflR* and *aflJ* genes work in concert in regulating aflatoxin biosynthesis in *A. flavus* and *A. parasiticus*. The *aflJ* gene has not been identified on the *A. nidulans* sterigmatocystin gene cluster, suggesting that the toxin synthesis between these fungi may be regulated somewhat differently.

(g) Nitrate added as the sole nitrogen source completely inhibits aflatoxin production in *A. flavus* and *A. parasiticus*, but ammonium supports it. The opposite is true for sterigmatocystin production in *A. nidulans*, i.e., nitrate is utilized as sole nitrogen source by this fungus for sterigmatocystin production (1, 2). This also suggests that there may be regulatory differences between these fungi.

It has also been demonstrated in our lab that the AREA protein produced by the major nitrogen regulatory gene binds to the intergenic region between *aflJ* and *aflR* in *A. parasiticus* (5). This information may be significant in the understanding of the regulation of toxin synthesis in the aflatoxigenic fungi.

(h) The results from our lab have demonstrated that fungal development and toxin biosynthesis may be regulated by a common genetic locus in *A. flavus* and *A. parasiticus* (6). Texas A&M scientists (N. Keller and T. Adams) have identified a locus in *A. nidulans* that is responsible for fungal development and may also control sterigmatocystin synthesis. The equivalent regulator is yet to be identified in *A. flavus* and *A. parasiticus*. But with the significant pool of developmental genes identified in *A. nidulans*, it may be easier to understand the correlation between fungal development and toxin synthesis in *A. flavus* and *A. parasiticus*.

A detailed understanding of HOW and WHY aflatoxins are made by *A. flavus* and *A. parasiticus* and an understanding of the molecular link between regulations of fungal development and toxin synthesis will provide insights into developing aflatoxin elimination strategies. It may be possible to not only prevent the fungus from producing toxins, but also affect fungal survival itself.

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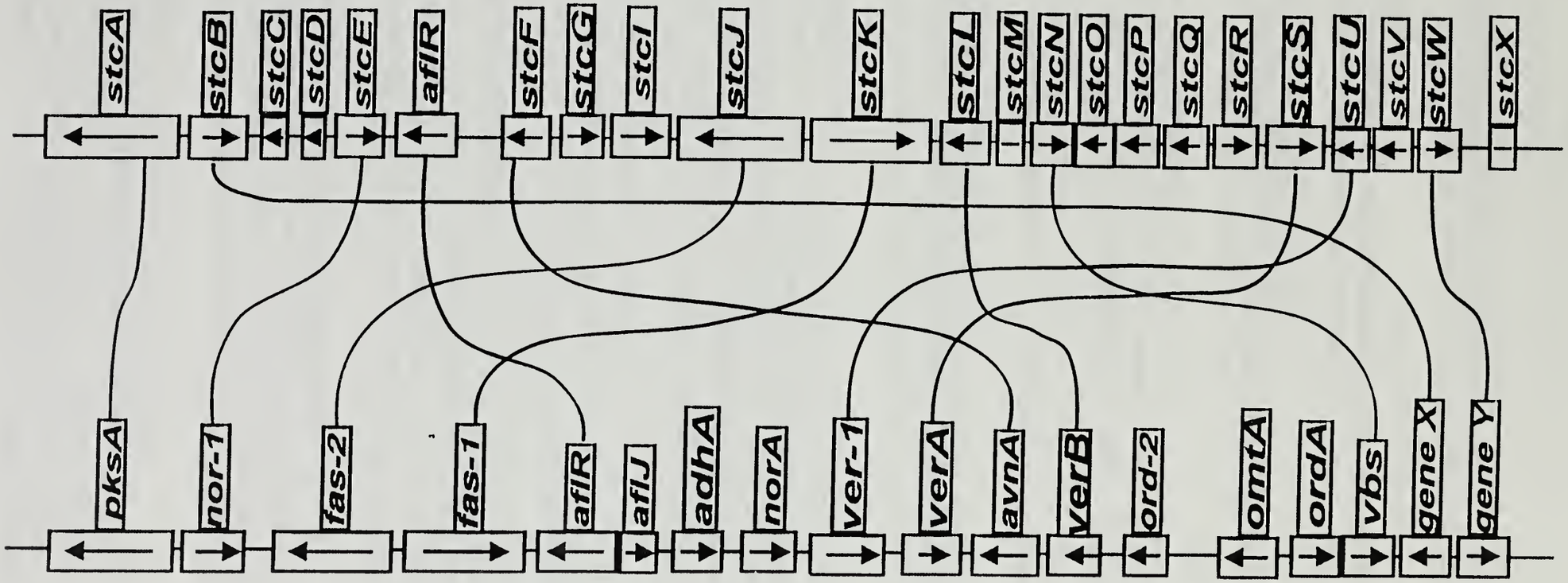
Fig. 1 . Comparison of aflatoxin & sterigmatocystin pathway gene clusters in Aspergillus spp.

A. nidulans



A. parasiticus & A. flavus

10 kb

IMMUNOCHMICAL STUDIES ON REGULATION OF AFLATOXIN FORMATION

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During the last few years, our effort was directed to use immunochemical methods as a tool in studying factors that regulate aflatoxin formation. The objective of these studies is aimed at searching bioactive agents for inhibiting fungal infestation and aflatoxin formation, with the hope that these factors could be cloned into the crops.

A. Approaches:

To reach the objective, our approaches involve: (1) generating specific antibodies against the key enzymes and regulatory proteins involved in the biosynthesis of aflatoxin; (2) developing sensitive immunochemical methods, including ELISA and Immunoblot, for the detection of these enzymes and proteins; (3) using these techniques in combination with other molecular-probes in understanding the mechanism of key factors/bioagents involved in toxin formation; (4) isolating and characterizing such factors. Over the years, the roles of several key enzymes and the regulatory protein, AFLR, involved in aflatoxin biosynthesis have been extensively studied with the availability of antibodies and specific immunochemical methods in our lab. These antibodies have been used in the Western blot and ELISA to monitor the expression and formation of these enzymes and proteins by various *Aspergilli* under various environmental and nutritional conditions. Because AFLR protein plays a key role in regulating aflatoxin production, understanding the role of AFLR in non-aflatoxigenic strains can provide mor information on how to control the aflatoxin formation in producers. Most of our attempts are focused on applying the immunochemical methods used for characterization of AFLR protein to identify the toxin-inhibition factors in non-aflatoxigenic fungal species and in corn inbreds resistant to aflatoxin formation.

B. Research Progress:

1. Characterization of AFLR protein in various *Aspergilli:* Western blot, ribonuclease protection assay (RPA) and ELISA analyses of selected aflatoxigenic *Aspergilli* (i.e. *A. parasiticus* and *A. flavus*) and non-aflatoxigenic *Aspergilli* (i.e. *A. flavus*, *A. sojae* and *A. oryzae*) species revealed that both *aflR* mRNA and AFLR protein were present in all the examined species; however, the *omtA* transcript, encoding a 42 kDa sterigmatocystin-*O*-methyltransferase, involved in the latter stages of the aflatoxin biosynthetic pathway, was not detected in any of them. AFLR in *A. oryzae* was found to be regulated by carbon source and temperature similar to the regulatory profile of AFLR in *A. parasiticus.* Sixty fungi isolated from Japanese fields were also screened by Western blot using anti-AFLR antibodies. Protein extracts from *A. nomius*,

non-aflatoxigenic *A. tamarii* and *A. caelatus* could be recognized by anti-AFLR antibodies showing a specific individual band. However, the size of the recognized protein in *A. nomius* preparations was around 58 kDa, which was larger than the expected size of AFLR (47 kDa) in other *Aspergilli.*

2. Identification of the factor(s) involved in the inhibition of aflatoxin formation in non-aflatoxigenic *Aspergilli*: protein extracts obtained from aflatoxigenic *A. parasiticus* NRRL 2999, non-aflatoxigenic *A. caelatus* 93SZ5 and *A. oryzae* NRRL 451 mycelial mass were partially purified through ultracentrifugation, hydroxyapatite (HA) chromatography and DEAE-cellulose chromatography. Electrophoretic mobility shift assay (EMSA) was then used to study the DNA (oligonucleotide containing specific sequence TTAGGCCTAA) binding ability of these partially purified protein extracts. The results of EMSA showed that protein-DNA binding complex was only found in HA column- purified protein extracts obtained from *A. parasiticus*, but not in protein extracts obtained from *A. caelatus* and *A. oryzae.* The protein present in the protein - DNA complex was identified to be AFLR protein by employing AFLR specific antibodies in an antibody competition assay.

3. Identification of resistant factors from aflatoxin-resistant corns : Seed of eight corn inbreds was kindly provided by Dr. Don White at University of Illinois. Most of them are known to have some levels of resistance to ear rot infection and/or aflatoxin production. Soluble corn extracts were extracted from the seed powder with phosphate-buffered saline solution, and then added into the GMS medium just inoculated with spores of *A. parasiticus* NRRL 2999. Fungal protein extracts were obtained from mycelia of three-day- shaking cultures and then subjected to Western blot analysis with anti-AFLR antibodies. Certain corn extracts did show the ability to repress AFLR formation on Western blot. However, the relationship between resistance to aflatoxin production and repression of AFLR production in corn extracts needs to be further investigated.

C. Selected Publications:

Yu, J., Cary, J. W., Bhatnagar, D., Cleveland, T. E., Keller, N. P. and Chu, F. S. (1993) Cloning and characterization of a cDNA from *Aspergillus parasiticus* encoding an O-methyltransferase involved in aflatoxin biosynthesis. Appl. Environ. Microbiol. 59: 3564-3571.

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POSTER PRESENTATIONS

PLANT METABOLITES ALTER ASPERGILLUS DEVELOPMENT

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The ratio between sexual and asexual development in *Aspergillus nidulans* appears to be dependent on linoleic acid-derived pheromones called psi factors which are structurally similar to products of the plant lipoxygenase (LOX) pathway. Linoleic acid and two LOX derivatives, 13 -hydroperoxylinoleic and 9-hydroperoxylinoleic acid mimicked psi activity in a concentration-dependent manner. Sexual development was promoted at 0.1 mg concentration (mimicking psiB and psiC activities) whereas asexual development was promoted at 1.0 mg concentration (mimicking psiA activities). We propose that seed fatty acids could be mimicking and/or interfering with endogenous *Aspergillus* pheromones and thus directing developmental processes in the fungus and that an *Aspergillus* lipoxygenase-like enzyme may be necessary for psi biosynthesis. In a previous work we reported that mycotoxin gene expression in *Aspergillus* was affected by these same LOX products (1). Our results suggest that LOX metabolite effects were not on mycotoxin genes directly but indirectly through effects on fungal growth. The information generated from this work could contribute to the design of control strategies to reduce mycotoxin biosynthesis and survival of seed-colonizing aspergilli.

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EFFECT OF WALNUT CONSTITUENTS ON *ASPERGILLUS* GROWTH AND AFLATOXIN PRODUCTION

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Aflatoxin contamination in pistachios, almonds, and walnuts is more prevalent in insect damaged kernels. *Aspergillus flavus* is a weak plant pathogen and damage to natural barriers to fungal contamination in tree nut kernels, such as cuticular layers and seed coat, enhances *A. flavus* colonization (2). Undamaged pistachio hulls are resistant to *A. flavus* colonization while damaged hulls are easily colonized; however, similarly damaged and inoculated walnut hulls showed no *A. flavus* colonization.

Naphthoquinones found in walnut hulls are known inhibitors of fungal growth (3). Four of these naphthoquinones were tested for their ability to inhibit *A. flavus* growth in potato dextrose agar. Fungal growth was completely inhibited by the following compounds: 1,4-naphthoquinone at 200ppm, 5-hydroxy-1,4-naphthoquinone (juglone) at 100ppm, and 2-methyl-1,4-naphthoquinone and 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin) at 50ppm. Both the 5-hydroxy and 2-methyl substituent on the naphthoquinone skeleton increased the growth inhibition activity. Aflatoxin production by *A. flavus* was not inhibited at naphthoquinone levels that did not affect growth.

Microscopic analysis revealed that these naphthoquinones disrupted the germination process. *A. flavus* spores normally begin to clump several hours after inoculation in potato dextrose broth and will begin to sprout germ tubes within 8 hours. In the presence of napthoquinones *A. flavus* spores exhibit neither the clumping behavior nor germ tube formation even after 2 weeks.

Inhibition of spore germination would explain the lack of fungal growth on inoculated damaged walnut hulls. Walnut hulls contain varying amounts of these naphthoquinones depending on the species (1) and seasonal variation. Additional napthoquinones present in walnut hulls (1) will be isolated to further examine the structure-activity relationship of these compounds as inhibitors of *A. flavus* spore germination.

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HOST-PLANT VOLATILES PREFERENTIALLY ENHANCE THE ATTRACTANCY OF THE SEX PHEROMONES OF MOTH PESTS ASSOCIATED WITH *ASPERGILLUS* INFECTION OF TREE NUTS

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Because insect feeding damage provides an avenue for *Aspergillus* invasion and infection of tree nuts, then fundamental to the elimination/reduction of aflatoxin is the development of effective control and pest management practices against moth pests. Our goal is to develop novel pest controls that utilize "semiochemicals," both host-plant volatiles (HPVs) and insect sex pheromones that evoke moth attraction. Current pheromone-based mating disruption control needs improvement in attraction efficacy.

Our hypothesis is that HPVs identified from non-nut, host-plants (*e.g.*, peaches, apples) will enhance or synergize the attractancy of moth sex pheromones when in a nut orchard context. Female moths release pheromone and mate while perched on their host-plants. Thus, pheromones and HPVs always share the same environmental context and might be adaptively associated in moth behaviors.

Experimental approach is to: 1) identify odor of nut and host fruit trees, and 2) field bioassay HPVs' inherent attractancy and synergism with pheromone. A dual-choice, paired-pheromone trap design was used to assess attraction and preference of males to: a pheromone only trap *vs.* a pheromone + HPV trap.

Coevaporation of HPVs with pheromones was found to increase by 1.3X to 3.6X the pheromones' attractancy to/preference by male moths. 40+ blends and 50+ individual HPV compounds were tested. The most active HPV synergists were: methyl octanoate (a peach volatile) for the peach twig borer (in almonds); and walnut monoterpenes [*p*-cymene, γ-terpinene, (*E*)-β-ocimene], apple/pear alcohols (butan-1-ol & 3-methylbutan-1-ol), and a blend of C-10 esters for the codling moth (in walnuts). Expression of HPV synergism of pheromones was dependent on the seasonal maturity state of the nut crop: being effective spring to mid-summer, non-effective during husk-split thru harvest phases of mid- to late-summer, while again effective in the post-harvest fall.

This synergistic enhancement by HPVs should enable sex pheromones to be more effective in control strategies for these tree nut pests in the spring and early summer. Controlling pest populations in the early season is critical if they are to be managed at levels below economic injury to insure the reduction of *Aspergillus* infections and aflatoxin in harvested nut commodities.

REPRESSION OF THE GUS REPORTER GENE OF AFLATOXIN BIOSYNTHESIS BY PLANT SIGNAL MOLECULES

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Infection of wounded tree nuts, peanuts, cotton and corn by *A. flavus* results in high levels of aflatoxin contamination hazardous to human health. The domestic and export markets of food products presently allow a maximum level of aflatoxin contamination to be 20ppb. Even very low levels of infection of the harvested food crops by *A. flavus* can significantly result in aflatoxin levels above these mandatory standards. In a worst-case scenario, the commodity may be banned from domestic and exporting markets. The presence of this microbial toxin reduces the quality and value of the crops and thereby directly affecting the economic return to the growers and processors.

Screening for natural compounds inhibiting aflatoxin biosynthesis in *A. flavus* has been a high priority research goal. The knowledge gained can be applied to develop strategies in plant breeding program. Also the natural plant compounds identified can be used in the food processing industry as additives to inhibit aflatoxin production by *A.flavus* during storage.

The phenolic signal molecules, acetosyringone, syringaldehyde and sinapinic acid,have recently been demonstrated in our laboratory to inhibit aflatoxin production by *A. flavus*. Accumulation of norsolorinic acid in a NOR mutant of *A. flavus* was also inhibited. We have applied the GUS reporter assays to elucidate the mechanism of inhibition. *A. flavus* strains 656-2 GAP12-19 (nor 1::GUS) and 656-2 GAP 13-22 (ver 1::GUS) were provided by G. A. Payne and J. L. Flaherty of North Carolina State University.

The three assays used for GUS activities to evaluate the inhibitory effects of these chemicals are: (1)a histological assay using x-Glu as a substrate to produce a blue precipitation as a qualitative indication of GUS gene expression; (2) a sensitive fluorescent assay using MUG as substrate for quantifying the extent of GUS gene expression; (3)a low affinity substrate assay using ONPG in the reaction to generate a yellow color. When 656-2 GAP 12-19 and 656-2 GAP 13-22 were cultured in media containing each phenolics at concentration from 1mM to 4mM, the GUS activities in these two fungal strains were reduced compared to controls without any phenolics in the media. The results of the assays suggest that these phenolic molecules inhibit transcription of *nor* and *ver* genes.

A MAIZE KERNEL TRYPSIN INHIBITOR IS ASSOCIATED WITH RESISTANCE TO *ASPERGILLUS FLAVUS* INFECTION

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Maize genotypes resistant to *A. flavus* had generally higher extractable protein content compared with susceptible genotypes when dry kernels were extracted using pH 2.8 buffer. The profile of protein extracts revealed that a 14 kDa protein is present in relatively high concentration in kernels of five resistant maize genotypes, but is absent or present only in low concentration in kernels of three susceptible ones. The N-terminal sequence of this 14 kDa protein showed 100% homology to a maize trypsin inhibitor. The association of this trypsin inhibitor protein with resistant maize genotypes may be related to their resistance to *A. flavus* infection and subsequent aflatoxin contamination.

Purified trypsin inhibitor protein from resistant varieties and from *E. coli* over expressing it demonstrated *in vitro* inhibition of fungal growth of not only *A. flavus*, but also all eight other fungi we have examined. The trypsin inhibitor concentration required to achieved 50% inhibition of fungal hyphal growth (IC_{50}) ranged between 33 to 102 μg/ml. This is the first demonstration of antifungal activity of a maize 14 kDa trypsin inhibitor protein. Further, the inhibition of fungal growth in PDA medium by trypsin inhibitor can be reversed by adding α-amylase in the bioassay. This result suggests that inhibition of fungal growth may be due to the limited availability of carbon source. This hypothesis was supported by the study using different carbon source in the bioassays, which showed that fungal growth was not inhibited by trypsin inhibitor protein at the same conditions when 1% gelatin or 5% glucose in A&M medium was used to replace PDA medium in the bioassays.

POTENTIAL ROLE FOR STORAGE PROTEINS AND SUGARS IN OILSEED SUSCEPTIBILITY TO AFLATOXIN CONTAMINATION

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The value of oilseed crops is diminished by aflatoxin contamination caused by the fungus *Aspergillus flavus*. Since seed storage proteins and storage carbohydrates comprise a significant proportion of seed dry weight in both corn and cotton, an investigation was undertaken to determine the effects of these seed components on fungal growth and aflatoxin production. A defined fungal medium (DFM) containing sucrose (50g/L) as the sole carbon source and sodium nitrate (3g/L) as the sole nitrogen source was used. Fungal cultures were grown for 5 days at 30°C; aflatoxin production was quantified with thin layer chromatography. Cultures grown in a medium containing sucrose with either cottonseed storage protein (CSP) or zein, but lacking nitrate, had higher aflatoxin production than cultures grown in DFM. Toxin production was strongly correlated (r^2=0.96, $P<0.05$) with initial concentration of storage protein. In addition, supplementation of DFM with soybean seed protein (1-20 g/L) stimulated aflatoxin production above DFM control levels. Either starch or the cottonseed storage trisaccharide raffinose was capable of supporting aflatoxin production as a sole carbon source. Toxin production was highly correlated with carbohydrate concentration (r^2=0.99, $P<0.05$). When raffinose was utilized as a carbon source (80 g/L) and CSP as the sole nitrogen source, increased aflatoxin and biomass were observed with increasing CSP concentration.

Whole cottonseed was ground into a meal for use as carbon/nitrogen source in fungal fermentations. This ground seed preparation was extracted with hexane (petroleum ether) to remove storage lipids (33% by weight). The resulting whole seed meal was both water-extracted and salt-extracted (10% NaCl) to produce meal preparations with different carbon/nitrogen compositions. Whole ground seed which was not hexane-extracted produced 280-fold more aflatoxin than meal which was lipid-extracted, suggesting that cottonseed lipids can function as an accessible carbon source for aflatoxin production. Ground seed extracted with both hexane and water produced 85-fold more aflatoxin than non-water-extracted meal (lipid-extracted), suggesting the presence of a water-soluble inhibitor in the whole seed meal preparation. A raffinose supplement (1.4% w/v) to the water-extracted meal stimulated aflatoxin production 3-fold, indicating that accessible carbon is limiting in this meal preparation and raffinose is capable of fulfilling this role. Aflatoxin production in the salt-extracted meal incubations did not differ significantly from that observed in water-extracted meal incubations, suggesting that cottonseed components other than raffinose and CSP can support some level of aflatoxin biosynthesis.

Effects of seed components on aflatoxin production will be discussed. The results suggest that both storage proteins and storage carbohydrates influence the vulnerability of oilseed crops to aflatoxin contamination.

β-CAROTENE INHIBITION OF AFLATOXIN BIOSYNTHESIS AMONG *ASPERGILLUS FLAVUS* GENOTYPES FROM ILLINOIS CORN

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Thirty-nine *Aspergillus flavus* genotypes (DNA finger-printing) isolated from corn grown in a field near Kilbourne, Illinois were evaluated for their sensitivity to β-carotene (50 μg/ml) inhibition of aflatoxin B_1 biosynthesis. Inhibition of aflatoxin was greater than 90% for 31 of the genotypes and >79% for 38 of the 39 genotypes. Five *A. flavus* strains (4 genotypes) isolated from molded raw peanuts, NRRL 3239, NRRL 3357, NRRL 6514, NRRL 6515 and NRRL 13135, produced greater quantities of aflatoxin than all 39 genotypes isolated from corn, and were less sensitive to β-carotene inhibition. *Aspergillus flavus* NRRL 3357 is commonly used as inoculum in variety trials for aflatoxin resistance. Isolate identity and sensitivity to potential inhibitors in corn can be critical in assessing corn resistance to aflatoxin. A hypothesis is offered that the cultivation of peanuts contributes to the accumulation of *A. flavus* clonal populations in field soil that produce consistently elevated quantities of aflatoxins and are less sensitive to naturally occurring aflatoxin inhibitors (e.g., carotenes, xanthophylls, etc.). Furthermore, in rotations with peanuts, corn should become infected with greater numbers of these more potent aflatoxin-producing *A. flavus* strains.

CHARACTERIZATION OF AN ALPHA-AMYLASE DEFICIENT MUTANT OF *ASPERGILLUS FLAVUS*

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Using a GUS-reporter assay we have detected an aflatoxin inducing activity in ground maize kernels colonized by *Aspergillus flavus*. Evidence suggest that alpha amylase produced by the fungus has a role in aflatoxin production in diseased maize kernels. We have hypothesized that the action of the fungal alpha amylase on kernel starch results in the production of fermentable sugars leading to an induction of aflatoxin biosynthesis. To test this hypothesis, we disrupted the alpha amylase gene *Amy1* of the aflatoxigenic strain 86-10D by site-directed mutagenesis. A transformant (T-150) having an *Amy1* disruption was identified by PCR methodology, and the mutation was confirmed by Southern hybridization. The rate of growth of T-150 and two control strains was identical on potato dextrose agar (PDA) medium On starch medium supplemented with 10 mM uracil and arginine, T-150 grew at 70% the rate of the control strains. T-150 grew on a medium containing 10 mM arginine and uracil as the sole carbon source (without starch), suggesting that arginine and uracil were responsible for the observed growth on starch medium. Iodine staining of the cultures grown on starch resulted in clear zones around the colonies of the control strains. In contrast, no clearing was detected around the T-150 colonies indicating that this mutant does not produce an extracellular alpha amylase. Also, extracellular alpha amylase activity was not detected when T-150 was grown on ground corn. After 8 days of incubation, T-150 appeared to grow in a viscous, starchy matrix with numerous starch granules. At the same time, the control strain grew more extensively, and the corn matrix was relatively clear with very few starch granules. These data confirm that the *Amy1* gene was disrupted in T-150. T-150 failed to produce aflatoxin when grown on rice kernels, ground corn kernels, coconut agar medium and PMSG (peptone mineral salt and glucose) medium. To determine whether the disruption of *Amy1* is responsible for the non aflatoxin phenotype, we have crossed T-150 with strain 118 (*tan, arg2, Tox*$^{+}$). We have obtained heterokaryons and putative diploids. The segregation of *Amy1* and aflatoxin in the progeny from this parasexual cross will be determined. We will also determine if progeny that are arginine prototrophs with a disrupted *Amy1* can grow on starch. The progeny will not require the addition of arginine to the culture medium.

INHIBITORY EFFECTS OF VOLATILE ALDEHYDES FROM AFLATOXIN-RESISTANT VARIETIES OF CORN ON *ASPERGILLUS PARASITICUS* GROWTH AND AFLATOXIN BIOSYNTHESIS

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Corn-derived volatile compounds have been previously found to affect growth and aflatoxin production in *Aspergillus parasiticus*. In this study, the effects of three of these compounds, *n*-decyl aldehyde, hexanal and octanal, on *Aspergillus parasiticus* were measured. These specific compounds were selected as representative volatiles because they have diverse effects on *A. flavus* growth and aflatoxin production. These effects were determined by measuring the amount of each aldehyde emitted by five aflatoxin resistant maize strains and three susceptible strains. Hexanal was emitted, to differing degrees, in both aflatoxin resistant and aflatoxin susceptible maize strains. Octanal was not emitted by any of the aflatoxin susceptible strains tested, but was emitted by three of the resistant strains. *n*-Decyl aldehyde was shown to have no effect on growth of *A. flavus*, but it reduced aflatoxin production by 10%. In addition, two volatile alcohols, 3-methyl-1-butanol and nonanol, and two volatile terpenes, camphene and limonene, have been shown to inhibit growth, stimulate aflatoxin production, and/or cause the formation of aerial hyphae in *A. parasiticus*. None of these volatiles proved to be ideal for the control of aflatoxin production, therefore a similar system was used here to screen volatiles that are produced by aflatoxin-resistant corn varieties, specifically *n*-decyl aldehyde, hexanal and octanal.

In this study, *A. parasiticus* radial growth was restricted least by n-decyl aldehyde and most by octanal. Treatments of 100 µl of both hexanal and octanal completely inhibited growth of the fungus. While the volatile compound *n*-decyl aldehyde had less of an effect on radial growth than the other volatiles, the *n*-decyl aldehyde treated colonies were predominated by uniquely aerial hyphae. These colony structures, when viewed using electron microscopy, were found to have more complex hyphae and significantly fewer conidiophores than the control and other aldehyde treatments. Furthermore, aflatoxin production by the fungus was reduced by *n*-decyl aldehyde and hexanal, but was stimulated by octanal. The results presented here indicate that all three volatile compounds reduce radial growth but only *n*-decyl aldehyde significantly inhibits aflatoxin biosynthesis in *A parasiticus*.

CHARACTERIZATION OF A CHITINASE FROM TEX6 INHIBITORY TO *ASPERGILLUS FLAVUS*

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Tex-6 is a corn inbred shown to be resistant to *Aspergillus flavus* pathogenesis (1). Our lab has developed an assay which is capable of quantitatively measuring fungal growth inhibition (2). Previous results have shown that Tex6 contains two inhibitory activities, one that inhibits fungal growth and the other that inhibits aflatoxin formation, but not fungal growth. We have focused on the purification of the growth inhibitory compound. Inhibitory activity in Tex-6 after gel filtration chromatography is associated with two bands on SDS-PAGE (1). The lower band reacts with antibodies to zeamatin, an antifungal compound identified in corn seeds. Partial peptide sequence of the upper band revealed significant similarity to a corn chitinase. Tex-6 extract also was found to test positive on a chitin plate clearing assay. We are continuing to purify this chitinase and are using the chitin analog MU-NAG to follow chitinase activity during the purification procedure. Data to date suggests that the majority of the antifungal activity in Tex-6 corn is associated with this chitinase activity. At this point, it is not clear if this is a yet unreported chitinase or if resistance in Tex6 is the result of a known chitinase being expressed at higher levels in Tex6 than other inbreds. The possibility also exists that the chitinase acts in conjunction with another compound in Tex6 to increase its toxicity.

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CROP RESISTANCE--GENETIC ENGINEERING

PANEL DISCUSSION

PANEL DISCUSSION TITLE: Enhancing Crop Resistance by Genetic Engineering of Crops

PANEL MEMBERS: Nancy Keller (Chair), Arthur Weissinger, Kanniah Rajasekaran, Caryl Chlan, Peggy Ozias-Akins, Abhaya Dandekar and Ray Bressan

SUMMARY OF PRESENTATIONS: Several trends were apparent in this session of engineering plants for aflatoxin resistance. Most significant was the move to use model systems to test the efficacy of anti-fungal genes. Others were the identification of tissue specific promoters and understanding the mechanistic nature of anti-fungals; the latter studies suggest that manipulation of fungal signal transduction systems may hold promise to control *Aspergillus* inoculum and aflatoxin production.

Problems to address include (1) improved Western analysis for all transgene-encoded proteins and (2) identification of stronger promoters for enhanced transgene expression as there is some evidence that transgenes are being expressed but not at a sufficient level to provide resistance (Author notes that translation stability and protein stability may need to be addressed in the future). Attention should also be directed to the phenomenon of gene silencing which can interfere with gene expression.

Use of Model Systems.
The two plant models used were tobacco (Weissinger group, Rajasekaran group, Chlan group) and Arabidopsis (Chlan group). The fungal models were *Cercospora arachidicola* (Weissinger group), *Fusarium moniliforme* (Rajasekaran group), *A. nidulans* (Keller group, Bressan group) and yeast, *Saccharomyces cerevisiae* (Bressan group). These models were employed as the aflatoxin susceptible crops, especially peanut and cotton, are hard to transform and the aflatoxigenic fungi, *A. flavus/A. parasiticus*, are sometimes difficult to work with. Ultimately any control program must have a strategy that works in the target crop against the target fungi, but these use of alternatives may point to the strategies most likely to succeed.

Current Anti-fungals used in Plant Transformations.
The antifungals favored for use include peptidyl membrane interactive molecules which are based on the structure of cecropins (Weissinger group). The MIM D5C gene was successfully introduced into peanut and transformed R0 plants showed enhanced resistance to *Cercospora arachidicola* but the trait was not passed to R1 progeny. Tobacco transformed with this gene showed resistance to fungal pathogens in a detached leaf assay but not in the field.

The Rajasekaran group focused on putting D4E1 (synthetic peptide), a haloperoxidase gene and a trypsin inhibitor gene into cotton and tobacco. Preliminary studies suggested that D4E1 transformed tobacco showed some resistance to *Fusarium*; however D4E1-transformed cotton did not. Tobacco has also been transformed with the haloperoxidase gene and the trypsin inhibitor gene and will be examined for resistance to *A. flavus* this coming year.

Chlan's group described dramatic improvements in the time required for cotton regeneration using an *Agrobacterium* mediated transformation system. However, it is such a labor intensive method that efforts will be focused on introducing antifungals into tobacco and Arabidopsis

over the next year. Several antifungal genes have been successfully introduced into Arabidopsis and the plants will be screened for fungal resistance in the coming year.

In Georgia, Ozias-Akins' group has been able to initiate the first field trial of transgenic peanuts (Cultivar Marc-1 carrying crylA(c) from *Bacillus thuringiensis*, which inhibits feeding of foliage by the corn earworm); data is currently being analysed. Other studies in progress include assessing various promoters (vegetative storage protein promoter) and the phenomenon of gene silencing. Peanuts are currently being transformed with the lytic peptide D4E1 driven by the ubiquitin promoter, the tomato anionic peroxidase gene driven by CamV35S, and the soybean lipoxygenase 1 gene (lox1) driven by the carrot DC3 promoter.

The Keller group is cooperating with Ozias-Akins and Cary et al. to put lox1 into peanut and cotton respectively. In a cooperative study with Pioneer Hibred, lox1 driven by the ubitiquitin promoter was inserted into corn. Preliminary laboratory studies suggest that the protein is expressed in seed and that less aflatoxin is produced in these transgenic seed. However, Western analysis must be improved to ascertain presence of Lox1 and field tests performed to support or refute the lab data.

Walnut somatic embryos have been successfully transformed by McGranahan et al. using an *Agrobacterium*-based system. Selection was on kanamycin medium with tests for GUS activity. GUS was successfully driven by either the 35S or ubiquitin promoters. Several trees have been generated from this transformation scheme.

Mechanistic Nature of Anti-fungals.
Soybean lipoxygenase 1 is an enzyme that uses linoleic acid as a substrate to generate oxygenated hydroperoxy-linoleic acids. Experiments by Keller's group showed that these products effect *Aspergillus* development by acting as sporogenic molecules that influence asexual, sexual and sclerotial production. As aflatoxin and sterigmatocystin production is linked to fungal development through a shared signal transduction system, it is thought that the effectiveness of lipoxygenase products lies in their ability to alter fungal development, perhaps through this same signal transduction system.

The Bressan group has shown that the antifungal agent osmotin hastens cell death in *Saccharomyces cerevisiae* by weakening cell wall barriers. Cell wall changes are induced via a yeast signal transduction system. Mutation of Sst2, one of the genes in this signal transduction system, leads to super-sensitivity to osmotin. A mutation in the *A. nidulans* homolog of Sst2, flbA, leads to *Aspergillus* sensitivity to osmotin. (Authors note: Interestingly, this same gene, flbA, is one of the signal transduction genes required for both fungal development and aflatoxin/sterigmatocystin production in *Aspergillus* such that *Aspergillus* does not sporulate or produce mycotoxins when flbA is mutated.)

SUMMARY OF PANEL DISCUSSION: Charles Woloshuk asked Nancy Keller where lipoxygenase would be expressed in cell. She replied that in plant cells, some LOX are expressed in chloroplast and presumably in cytosol in other cells such as seed cells although she was not sure. Hal Gardner thought this was also likely to be the case. Woloshuk also asked if an increase in 13-HPODE could be detected in transformed corn seed. Nancy Keller said this has not been done yet, but said it might be hard to detect an increase as 13-HPODE will be metabolized by the plant enzymes.

Ray Bressan suggested that aflatoxin was a signal to plants to turn on their defense systems. Deepak Bhatnagar asked how this hypothesis would fit in with the finding that some *A. flavus* isolates were atoxigenic and some not. One would think that more *Aspergillus* strains would be atoxigenic. This led to a lively discussion that was not conclusive but certainly entertaining. Charles Woloshuk and Ray Bressan will be checking to see if aflatoxin elicits plant response genes to check this hypothesis.

Kitty Cardwell had some concern that transgenic plants might express an anti-insecticidal protein in the pollen which would effect the pollinators or other non-target organisms. Pat Dowd knew of studies where transgenic plants expressing the Bt crystal protein had no effect on predatory insects.

Another concern was that transgenic proteins could act as antigens to humans and other consumers such that people/animals could develop allergies to the transgenic foods. These are serious concerns and everyone agreed steps would have to be taken to assess these concerns. Transformation with genes encoding proteins already found in seeds (like the lipoxygenase gene) would more likely avoid this last concern. The effects of novel types of protein (Demeter peptides) would have to analyzed for such affects.

A general concern was the reaction of the European Community to transgenic plants. It was felt that the U.S. government had to interact with European officials to work on better acceptance of transgenic plants.

PLATFORM PRESENTATIONS

ENGINEERING PEANUT FOR ENHANCED RESISTANCE TO *A. FLAVUS*

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Our goal is to develop a transgenic peanut lines expressing anti-fungal peptides. We have chosen to concentrate our efforts on transformation with genes encoding Peptidyl MIMs (Membrane Interactive Molecules), synthetic peptides based on the structure of naturally-occurring cecropins. Early experiments with these peptides *in vitro* demonstrated that they disrupt fungal mycelia and retard spore germination at concentrations as low as 5 uM. Experiments have been undertaken to transform the Virginia peanut cultivar NC 7 with MIM D5C (MW 3.3 KD) fused with a signal peptide derived from the vicillin gene of *Pisum* (D5C sec.). This peptide, which is active against *A. flavus* at concentrations of 10 uM, is expected to be secreted from the plant cells and accumulate in the intercellular spaces. Encroaching fungi would then likely encounter the peptide in the course of infection, limiting subsequent growth and reducing severity of the infection. Aflatoxin contamination of peanut might be reduced if growth of *A. flavus* could be retarded in this way.

We have transformed NC 7 with D5C sec *via* microprojectile bombardment of embryogenic cultures and selection with hygromycin by previously published protocols. Inheritance of the transgene has been demonstrated in R_1 progeny. Presence of the transgene has been demonstrated by PCR and Southern-blot analysis of genomic *DNA*.

A procedure was developed which allows reliable inoculation of peanut plants with *Cercospora arachodichola*, a fungal pathogen that is also retarded by D5C *in vitro*, at protein concentrations similar to those required to retard the growth of *Aspergillus*. It serves as a useful model for fungal resistance in the in greenhouse since it is easier to manipulate than *A. flavus*. Some transgenic lines (R_0) carrying D5C exhibited enhanced resistance to the pathogen. This effect was lost in the R_1 progeny, however. No D5C mRNA was detected by RNA hybridization analysis (northern blot) probed with the D5C coding sequence.

Tobacco cv. NC1013-35 was also transformed with MIM D5C sec. Doubled haploid R_1 progeny were shown to carry and produce transcript of the D5C transgene. Plants were resistant to *Rhizoctonia* and *Phytophthora* in a detached leaf assay, but did not exhibit enhanced disease resistance in greenhouse or field trials.

Improved immunoblot (western) protocols have been developed which are sufficiently sensitive to detect pure D5C peptide at the level of about 47 ug per sample, but failed to detect peptide in extracts from any transformed plant. This test is not sensitive enough, however, to detect peptide at the far lower concentrations previously found to retard *A. flavus in vitro*.

Goals for the coming year include development of more sensitive western procedures that will permit testing of the several hypotheses consistent with data obtained from first-generation transformants. Further, new transformations have begun using an improved vector in which D5C is driven by the highly active potato Ubi 7 promoter. Finally, a new gene, D2A2, with far higher specific activity against *A. flavus* will also be introduced into peanut.

AGROBACTERIUM-MEDIATED TRANSFORMATION AND ANALYSIS OF COTTON EXPRESSING ANTIFUNGAL PEPTIDES

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Several antifungal factors are being evaluated for their activity in transgenic cells and callus cultures of cotton and tobacco. These antifungal factors include D4E1, a linear, synthetic peptide and a haloperoxidase gene construct of *Pseudomonas* origin.

Transformation with the antifungal peptide D4E1

Agrobacterium-mediated transformation of cotton (variety Coker 312) has been accomplished with the antifungal peptide D4E1. Thus far, transformation of cotton has been carried out with *Agrobacterium* vectors containing the D4E1 gene driven by 35S:CaMV, ubiquitin 3 or ubiquitin 7 promoters. Several transformed callus cultures, identified by Southern hybridization of D4E1 - PCR products, have been induced to become embryogenic from which transgenic cotton plants are being developed. In vitro bioassays were conducted to test antifungal activity of transformed cotton callus cultures against germinated conidia of *Fusarium moniliforme*. Previous studies have indicated that the purified synthetic peptide D4E1 inhibited the further development of germination conidia of *F. moniliforme* at concentrations as low as 1.5 um. Preliminary bioassays performed with randomly selected, non-embryogenic transformed cotton callus cultures showed little or no antifungal activity against *Fusarium* conidia. However, bioassays using extracts from tobacco tissue transformed with the same D4E1 constructs demonstrated growth inhibition of *F. moniliforme* conidia by 50 to 80% compared to non transformed tobacco controls. Transformation experiments to date with both cotton and tobacco indicate the need for achieving higher levels of antifungal expression of D4E1 by the use of strong promoters (see abstract by Cary et al.) and analysis of the fate of D4E1 *in vivo* or *in planta*.

Transformation with the haloperoxidase gene construct

Potential of haloperoxidase (HPO) as an antifungal agent has been demonstrated using transgenic tobacco model system. Integration and expression of haloperoxidase in transgenic tobacco has been demonstrated by Southern and Western analyses. Halogenating activity due to the presence of haloperoxidase enzyme activity in transgenic tobacco has also been documented. The mode of action of haloperoxidase utilizes the fact that the plants generate H_2O_2 in response to invading pathogens. In the presence of haloperoxidase, enzymatic halogenation occurs which results in the formation of two potent microbicides - hypochlorous acid and peracetic acid. Bioassays of tobacco extracts for efficacy against *A. flavus* and transformation of cotton with haloperoxidase are in progress.

COTTON TRANSFORMATION AND A NEW SYSTEM TO ASSAY THE EFFICACY OF POTENTIAL ANTIFUNGAL GENES

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Aspergillus flavus is a fungal pathogen that infects cotton bolls. During its life cycle, the fungus can produce high levels of aflatoxin. Aflatoxin contamination of cotton seed decreases the value of this byproduct of the cotton industry. Since varieties of cotton that are naturally resistant to *A. flavus* are not available, we are attempting to develop varieties of cotton with increased resistance to *A. flavus* using genetic engineering techniques.

To develop strains of cotton using this approach, it is necessary to introduce new traits into cotton cells, select for those transformed cells, and regenerate those cells into whole plants. The process of introduction of new traits into cotton can be accomplished by several techniques including bombardment of the tissue with DNA coated particles, or transformation medicated by *Agrobacterium*.

We are using an *Agrobacterium* medicated transformation system to introduce new genetic material into cotton. Once cotton cotyledon tissue is incubated with solutions of *Agrobacterium*, the transformed cells are selected by incubating with compounds that test for the expression of a co-transformed marker gene. After selection, the transformed cells must be regenerated into cotton plants. This step is difficult, laborious, and rate limiting. Last year, we reported advances made in the earliest regeneration step - callus induction. Over the past year, we have seen dramatic improvements in our regeneration time course, efficiency, and reproducibility as a consequence of procedural modifications. We have now regenerated over 30 different plantlets (at least 2 leaves and 2 roots) from cotyledon tissue that was treated with three different potential antifungal gene constructs. These plantlets are being grown for future studies that include PCR and Southern analyses (to confirm integration of antifungal gene), western blot analyses (to determine expression levels of the gene) and bioassay studies (to determine efficacy of genetically engineered cotton extracts against different pathogens including *A. flavus*).

The optimized protocol allows us to regenerate plantlets from *Agrobacterium* treated cotyledon tissue in 6-7 months. This process is extremely labor intensive, and not well suited for rapidly screening potential antifungal genes to determine their activity *in vivo*. Therefore, we have been working with other plant systems to generate materials to test the *in planta* efficacy of these genes in a more rapid, less laborious system.

We considered two different systems for this purpose: tobacco leaf disk transformation and regeneration, and *Arabidopsis* vacuum infiltration. Although tobacco transformation and regeneration is an efficient process with which we are very experienced, it also requires regeneration of plants in tissue culture (labor intensive), even dwarf varieties of tobacco are

rather large (require a fair amount of greenhouse space), and the life cycle of tobacco requires several months (long time frame required to study seed specific gene expression).

Ideally, we need a test system that will enable us to study genes driven by promoters that direct expression in different tissues at different stages of development. Consequently, a plant with a short life cycle would be most appropriate to study gene expression in many different stages of ontogeny within a short time frame. *Arabidopsis* has a short life cycle (8-12 weeks) and new methods for the transformation of *Arabidopsis*[1,2] are simple, rapid, and do not require tissue culture. We have transformed *Arabidopsis* with several of the putative antifungal constructs and control plasmids to monitor the expression of genes in this system. These tissues are stably transformed, and express newly introduced genes based on pilot studies with a GUS construct. Bioassays of transgenic *Arabidopsis* tissues that contain putative antifungal genes are in progress.

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EXPRESSION AND INHERITANCE OF TRANSGENES IN PEANUT - GREENHOUSE AND FIELD STUDIES

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Transformation of peanut, *Arachis hypogaea* cv. Marc-1, was carried out with a synthetic *cryIA*(c) gene from *Bacillus thuringiensis*. Progeny (R1) from the primary transgenic plants were screened by in vitro feeding of foliage to corn earworm. Those individuals which showed zero larval survival, a <1.5 damage rating on a scale of 1-4, and a total larval weight of <5 mg were saved for a field test in summer, 1997. An APHIS approved field test was initiated on June 16 when R2 seedlings, from R1 families showing some resistant individuals, were transplanted to plots on a 30 cm spacing. Out of 493 seeds from 4 R1 families planted, 400 seeds (81%) germinated. Tomato spotted wilt virus was severe in 1997 throughout south Georgia, and 25% of the transgenic plants displayed symptoms of virus infection. Yields from the infected and stunted plants were very low or non-existent. Rainout shelters were placed over the field test 2.5 months after transplanting, and plants were infested twice weekly, for five weeks, with lesser cornstalk borer larvae. Pods were harvested 4 months after transplanting. Many showed visible damage from the lesser cornstalk borer including scarification and penetration. Some plants in transgenic lines had a large number of undamaged pods, although data on each pod have not yet been taken. Pods from each individual will be scored for surface damage, penetration, and lack of damage. Plants also are being screened for the presence of the gene by PCR.

Progenies (R2) of transgenic plants containing the β-glucuronidase (gus) gene driven by the vegetative storage protein gene promoter have been screened by PCR for the presence of the gene and by histochemical methods for the expression of the gene. The vsp-gus gene typically is strongly expressed in stems (near the vascular tissues), pod walls, and pollen. Individual pollen grains can be scored for gus activity, and the segregation patterns for individuals in different families ranged from no expression (either absence of gene or gene silencing), all grains expressing (one or more copies of the gene in each pollen grain), 50% of grains expressing (probably a single copy of the gene that is segregating during meiosis), 75% of grains expressing (possibly two unlinked copies of the gene segregating independently during meiosis), to 25% of grains expressing (difficult to explain without invoking gene silencing). Leaf material has been collected for DNA isolation and Southern analysis to determine copy number and segregation of unlinked copies. In a related study with transgenic plants containing the N-gene of TSWV, a putative case of gene silencing has been observed and was correlated with high copy number of the introduced gene. One line with only a single copy of the gene shows high expression of the N-protein and segregates 3:1 for presence of the gene.

More recent experiments are in progress to generate transgenic plants with the lytic peptide gene, D4E1 (driven by the ubiquitin promoter), the tomato anionic peroxidase gene, tap1 (driven by CaMV35S), and the soybean lipoxygenase gene, lox1 (driven by the carrot DC3 promoter). Covalently-linked genes, D4E1, tap1, and the hygromycin resistance gene have been introduced into 22 cell lines, 14 of which thus far have been regenerated to plants. Preliminary analyses by PCR show the presence of all three genes. Transgenic plants will soon be analyzed by Southerns, Northerns, Westerns and RT-PCR. Bombardments to introduce the lox gene also have been carried out and materials are under antibiotic selection.

GENETIC ENGINEERING OF TREE NUT CROPS FOR CONTROL OF AFLATOXIN
1997

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Genetic improvement is a long term approach to controlling aflatoxin contamination in affected crops. Classical breeding for resistance to the causal agent or its mode of entry or multiplication can be greatly enhanced by biotechnological approaches, such as asexual introduction of useful genes into cultivars or breeding lines. For the nut crops, walnuts serve as a model system for this technology because transformation and regeneration of transgenic plants has been accomplished and is relatively routine. The major emphasis of this project is now on engineering and field testing plants with resistance to insects that predispose nuts to infection by *Aspergillus flavus* and the resulting production of aflatoxin.

In October 1996 somatic embryos of lines CR1, CR2, CR3, 76-80xChico #12, 85-8x85-10 #1, and CRx85-8#2 were inoculated with *Agrobacterium tumefaciens* containing plasmids pDU92.710 or pDU96.3113. The embryos used were taken from actively growing cultures containing white embryos of varying stages from globular embryos to fully developed embryos which were already producing new embryos on their surfaces. No older, browning embryos were used. One to three plates of embryos were used for each treatment depending on the available material of each genotype and the amount of room in the container used. Initial embryos varied widely in size and were not individually counted.

Two treatment methods were employed. In the first, overnight bacterial cultures were centrifuged and resuspended to a density of 2.5×10^8 bacteria/ml ($A_{420} = 0.5$) in liquid Driver-Kuniyuki walnut (DKW) basal medium containing 100 uM acetosyringone. Embryos were immersed in this bacterial culture for about a half hour, removed to sterile filter paper to blot off excess liquid, and plated to the same medium solidified with Gelrite. A second set of embryos was treated identically except the bacteria were first held in an induction medium (IM) consisting of liquid DKW medium, 100 uM acetosyringone, 1 mM proline, and reduced sucrose (20 g/l) for 5 hours to stimulate virulence.

After 48 hours, all embryos were transferred to plates of DKW containing 100 mg/l kanamycin and 500 mg/l cefotaxime. Embryos were then transferred to fresh plates of the same medium at 2, 4, and 7 days after initiation and then transferred weekly.

Embryos which developed on kanamycin medium were tested for GUS activity and positive embryos were further multiplied and tested. Numbers of initial (E_0) embryos from which transgenic lines were derived are shown in Table 1. Transformation efficiency was not quantified. The objective was to obtain some transformants in each genotype. Success depended on the quality and quantity of the initial embryos used. CR1 and 85-8 x 85-10 #1 embryos were very white, dividing rapidly and available in abundance. The other lines were growing more slowly at the time the bacteria was applied and the amount of initial material was limited. Transgenic embryo lines were selected after two generations of multiplication on selection medium and GUS testing.

Table 1. Number of initial embryos producing transgenic lines for each plasmid and treatment.

Embryo Genotype	Plasmid				
	pDu92.710 (35S)		pDU96.3113 (ubi)		No Treatment
	Basal	IM	Basal	IM	Basal
CR1	3	5	4	3	-
CR2	-	-	-	2	-
CR3	-	-	-	-	-
76-80 x Chico #12	1	-	-	2	-
85-8 x 85-10 #1	6	3	2	5	-
CR x 85-8 #2	1	-	1	1	- -
Total	11	8	7	13	-

Embryos of selected GUS-positive lines were tested in insect feeding trials. Lines were chosen for testing with the goal of identifying, where possible, two lines of each genotype/plasmid combination. The tests were based on mortality of single codling moth larvae after 5-7 days of feeding on individual embryos. Thirty embryos of each line were tested and the embryo lines were characterized as Class A, B or C depending on percent mortality. The difference between promoters was not significant.

Table 2. Number of lines bioassayed, by plasmid and insect mortality class.

Embryo genotype	Plasmid					
	pDu92.710 (35S)			pDU96.3113 (ubi)		
	A*	B	C	A	B	C
CR1	7	1	3	6		
CR2	-	-	-	2		
CR3	-	-	-			
76-80 x Chico #12	-	1	-	3		
85-8 x 85-10 #1	4	-	-	3	1	1
CR x 85-8 #2	1	1	-	3		
Total	12	3	3	17	1	1

*A = 90-100%, B = 60-89%, C = 0-59% mortality. Controls averaged 35% mortality.

Trees have been produced from the Class A lines by germinating embryos, micropropagating the hypocotyls, and chip budding the resulting shoot buds to seedling rootstocks in the greenhouse. To date 140 trees have been produced (Table 3) for establishment in two field trials. Tree production is continuing. A dozen trees were fall planted in the field. Additional trees of sufficient size have been moved to a lath house to induce dormancy and to receive chilling before late-winter planting. Trees that need additional growth and trees budded this winter will be planted after danger of frost in the spring of 1998.

Table 3. Number of grafted trees produced.

Embryo genotype	Plasmid	
	pDu92.710 (35S)	pDU96.3113 (ubi)
CR1	47	63
CR2	-	14
CR3	-	-
76-80 x Chico #12	-	14
85-8 x 85-10 #1	-	-
CR x 85-8 #2	2	-
Total	49	91

OSMOTIN ACTS VIA SIGNAL TRANSDUCTION SUBVERSION SUMMARY

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The plant pathogenesis-related protein osmotin is an antifungal cytotoxic agent that causes rapid cell death in the yeast *Saccharomyces cerevisiae*. We show here that osmotin treatment weakens defensive cell wall barriers in yeast to increase its cytotoxic efficacy. These cell wall changes are induced via a signal transduction pathway that includes STE4, STE18, STE20, STE5, STE7, STE11, FUS3, KSS1, and STE12. Although all of these signal elements also participate in the mating pheromone response, neither pheromone receptor STE2, nor its associated G protein a subunit GPA1 was required for osmotin action. Phosphorylation of STE7 was rapidly stimulated by osmotin treatment preceding any changes in cell vitality or morphology. Mutation of SST2, a negative regulator of Ga proteins, resulted in super-sensitivity to osmotin.

LIPID METABOLITES ALTER *ASPERGILLUS NIDULANS* DEVELOPMENT

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The genetically accessible sp., *Aspergillus nidulans*, exhibits two reproductive stages: the first one is sexual resulting in the formation of a fruiting body called a cleisothecium and the second reproductive stage is asexual culminating in a mature conidiophore. The relative development of sexual or asexual structures in *Aspergillus nidulans* appears to be dependent on linoleic acid-derived pheromones called psi factor (Mazur et al., 1991). Psi factor is a mix of three hydroxylated linoleic acid molecules, psiA, psiB and psiC, where psiB and psiC promote sexual sporulation while psiA is antagonistic to psiB and psiC. We have found that some plant derived fatty acids, which present structural similarities to *A. nidulans* psi factor, also regulate the ratio between sexual and asexual development in a concentration-dependent manner. At high concentrations these plant fatty acids significantly promoted asexual development and simultaneously had an inhibitory effect on the sexual development of *A. nidulans* (mimicking the activity of psiA). At a 10-fold decreased concentration, these fatty acids showed the opposite effect (mimicking psiB and psiC activity). Due to the structural and functional similarities between these plant metabolites and psi factor, we propose that seed fatty acids could be mimicking and/or interfering with endogenous *Aspergillus* pheromones and thus directing developmental processes in the fungus. The relationship between fungal development and AF/ST production has been recently demonstrated in our lab (Hicks et al., 1997). Therefore, understanding the effects of natural fatty acids on fungal development could aid in the reduction of mycotoxin biosynthesis.

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POSTER PRESENTATIONS

CLONING AND CHARACTERIZATION OF A LIPOXYGENASE GENE FROM PEANUT

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Previous studies showed that lipoxygenase products inhibit spore germination of *Aspergillus*. We have reported that the 13-hydroperoxylinoleic acid (13-HPODE) product of soybean lipoxygenase reduced mycelial weight, decreased the level of expression of the aflatoxin (AF) pathway genes and mycotoxin biosynthesis in vitro. In contrast, 9-hydroperoxylinoleic acid (9-HPODE) had very little or no effect on growth and mycotoxin production in *Aspergillus* spp., but instead extended the time the AF gene transcripts were detected. These results indicate that lipoxygenases and their products could be important in controlling AF contamination of susceptible plants like peanut. To better understand the role of native lipoxygenases and their products during peanut-*Aspergillus* interaction, we have cloned and are currently characterizing Pn *lox1*, a lipoxygenase gene from an immature peanut seed cDNA library. Comparison of the deduced amino acid sequence of Pn *lox1* to available sequences in Gen Bank indicated high homology to soybean *lox2* gene. Pn *lox1* hybridized to an ~2.8 Kb transcript that was strongly expressed in immature seed. Inoculation of immature seed with *A. parasiticus* ATCC 98016 did not alter the level of Pn *lox1* transcript.

USE OF *ARABIDOPSIS* AS A MODEL SYSTEM TO TEST THE *IN VIVO* EFFICACY OF GENE PRODUCTS AGAINST *ASPERGILLUS FLAVUS*

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We are in the process of genetically engineering cotton using an *Agrobacterium*-mediated transformation system to generate transgenic cotton plants with improved resistance to *Aspergillus flavus*. Since this is a lengthy process, and various potential anti-fungal gene constructs have been made, we need to test the efficacy of these gene products in inhibiting the fungus prior to introduction into cotton.

Originally, tobacco was planned to be used for this study because it is a common model system for plant molecular research, and we already have the expertise needed for efficient transformation and regeneration of transgenic plants. However, in recent years *Arabidopsis thaliana* (L.) has been used more widely as a model system because of its greater advantages. The small plant has a short life cycle of only 4-6 weeks, and a single plant can be grown on as little as 1-2 cm^2. A very simple and efficient protocol for transformation of *Arabidopsis* by vacuum infiltration has been available since 1993[1].

We have implemented the *Arabidopsis* vacuum infiltration procedure in our study to test the potential anti-flavus gene constructs. Bent[2] and Green[3] protocols were used. The plants were grown in 4 inch pot covered with a screen. Primary bolts were clipped off to encourage growth of multiple secondary bolts. Six to eight days after clipping, when many secondary bolts were 1-5 cm long, the plants were inverted into *Agrobacterium* infiltration solution and infiltrated for 10-15 min at a vacuum of 20-23 inch Hg. They were then grown to seed harvest. The seeds were plated on MS medium containing kanamycin at 50 mg/l to select transformants and carbenicillin at 400 mg/l to control agrobacterial growth. The transformants, which were identifiable as dark green plants, were then transplanted to soil and grown individually to collect seeds.

Using this protocol, we have been working with a series of constructs and have obtained transformants from 5 different gene constructs that encode potential anti-fungal proteins, including a Demeter anti-fungal peptide, two osmotins, a protease inhibitor and a chitinase/osmotin dual construct. In the control experiment, PBI121 carrying the GUS gene was used to transform the *Arabidopsis* plants. The presence of the expected GUS marker gene in the transformants was confirmed by PCR amplification. The GUS expression was analyzed with the fluorescent assay of the protein extract or by histochemical staining of whole seedlings. We also have *Arabidopsis* plants that have been vacuum infiltrated with 3 other anti-fungal gene constructs, including a bean chitinase, a chitinase/glucanase dual construct and PGIP.

We are currently testing the transformed R_0 plants and their progeny for the presence of the gene introduced by PCR amplification or Southern Blot, and analyzing the expression level of the anti-fungal protein by Western Blot. Eventually we will assay whole plant responses to pathogen challenge. The results obtained from these model plant studies will allow us to determine how to manipulate the gene to express the anti-fungal compounds in appropriate tissues at levels that will be effective and identify constructs that are most likely to be effective in transgenic cotton.

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Green lab protocol for vacuum infiltration transformation of *Arabidopsis*. (Personal communication)

TRANSFORMATION AND ANALYSIS OF COTTON AND TOBACCO TISSUES EXPRESSING ANTIFUNGAL PROTEINS AND PEPTIDES

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Our goal is to transform commercial varieties of cotton with gene constructs encoding antifungal peptides/proteins to reduce aflatoxin contamination and the loss of lint yield and quality due to invasion by the fungus, *Aspergillus flavus*. We previously reported on the evaluation of several natural and synthetic proteins and peptides for antifungal activity *in vitro*. A synthetic peptide, D4E1 at 1.5 µM and 12.5 µM has been shown to completely inhibit the growth of germinating conidia of *A. flavus* and *Fusarium moniliforme*, respectively, and is fairly resistant to degradation by both plant and fungal proteases. A number of vector constructs utilizing a gene encoding D4E1 under control of the 35S CaMV promoter have been introduced into cotton cells by *Agrobacterium*-mediated transformation. We have also transformed these constructs into tobacco cells as a means of quickly determining if the peptide/protein is being expressed and if it is being expressed at efficacious levels. Bioassay of tobacco callus tissues transformed with the 35S CaMV-D4E1 construct demonstrated a 50-80% inhibition in the germination of *F. moniliforme* spores while transgenic cotton extracts demonstrated little if any inhibition. The level of inhibition demonstrated against *Fusarium* in the tobacco assays yielded an estimated concentration of 0.5-1.2 µM D4E1 which is approximately 10 fold less than required for inhibition of *A. flavus* spore germination (as determined by previous *in vitro* assays). These results indicated that the level of expression of the D4E1 gene under control of the 35S CaMV promoter was not great enough in either tobacco or cotton to effect inhibition of *A. flavus* spore germination (a conc. of ~12.5 µM). Therefore, a series of experiments were conducted to identify a promoter that could facilitate this required level of gene expression in cotton and tobacco. We evaluated different promoters in cotton by studying the expression of the marker gene, *uid A* (GUS), under the control of 35S CaMV, double 35S CaMV with a tobacco mosaic virus (TMV) enhancer sequence (Skuzeski et al. 1990 Plant Mol. Biol. 15:65), ubiquitin 3, ubiquitin 7, potato protease inhibitor II (PIN II), and the Gelvin "super promoter" with and without the TMV enhancer (Li et al. 1995 Plant J. 7:661), following biolistic-bombardment of cottonseed embryos. Results of fluorometric 4-methyl umbelliferyl-β-glucuronide (MUG) assays of bombarded embryos gave the following relative strengths for the promoters tested:

	d35S-TMV>	Gelvin-TMV>	Gelvin>	PIN II>	35S CaMV>	ubi 7, ubi 3
relative strength	*200*	*100*	*35*	*30*	*20*	*15*

These results indicated that future constructs should utilize the d35S CaMV-TMV enhancer promoter if the levels of D4E1 peptide needed for inhibition of *A. flavus* are to be achieved.

Plants generate hydrogen peroxide (H_2O_2) in response to invading pathogens. In the presence of the bacterial enzyme, haloperoxidase, enzymatic halogenation occurs which results in the formation of potent microbicides hypochlorous acid and peracetic acid. Tobacco plants were transformed with a vector expressing a bacterial haloperoxidase gene under control of the 35S CaMV promoter in order to examine the ability of this gene product to inhibit the growth of *A. flavus*. Western blots of callus tissue from putative tobacco transformants demonstrated that the haloperoxidase was being expressed. Activity assays (ability to chlorinate the substrate, monochlorodimedon) of plant extracts also demonstrated halogenating activity was present. We are awaiting the generation of enough transgenic plant material to undertake assays for the efficacy of haloperoxidase to inhibit the growth of *A. flavus*. Similar experiments with cotton are also in progress.

CROP RESISTANCE--CONVENTIONAL BREEDING

PANEL DISCUSSION

PANEL DISCUSSION TITLE: Enhancing Crop Resistance by Conventional Breeding of Crops

PANEL MEMBERS: Charles Woloshuk (Chair), Robert Brown, Thomas Gradziel, Corley Holbrook, Charlie Martinson, Gary Payne, Donald White, Donald Wicklow and David Wilson

SUMMARY OF PRESENTATIONS: The speakers reported on their efforts to understand resistance and susceptibility to *Aspergillus flavus* infection and aflatoxin production in plant genotypes derived from conventional breeding. A variety of innovative techniques were used, including X-ray analysis, reporter genes, and restriction fragment length polymorphism (RFLP).

Identifying resistance to *A. flavus* and aflatoxin production by conventional breeding relies on methods that accurately measure the resistant phenotype. Gradziel, Holbrook, White, Wicklow, Martinson, and Wilson reported on their research to identify resistant genotypes. Studies by White indicate that inbred Tex6 contain genes for resistance to Aspergillus ear rot. White presented data indicating that resistance genes are dominant and additive. White also presented his group's latest efforts to map resistance genes by RFLP techniques and to identify molecular probes for marker assisted selection of resistant genotypes. Wilson presented data showing that a mutant of *A. parasiticus*, which accumulates the aflatoxin pathway metabolites norsolorinic acid, can be used to assess aflatoxin production in infected corn kernels. Rating norsolorinic acid production by its red color, Wilson found excellent correlation with aflatoxin levels. This method should reduce the time and cost of scoring genotypes for resistance to aflatoxin production. Wicklow showed the potential use of bright greenish-yellow fluorescence (BGYF) to evaluate resistance in corn. Holbrook presented his group's progress in screening the peanut core collection for resistance to preharvest aflatoxin contamination. Gradziel reported on the use of X-ray analysis to evaluate the endocarp of almonds as a barrier to navel orangeworm (NOW). Preventing damage by NOW would effectively lower aflatoxin contamination. Martinson reported on the segregation of a hexane-soluble inhibitor of aflatoxin biosynthesis in mutagenized B73 and A632 inbred lines of maize.

The underlying biochemical reasons for resistance and susceptibility in host genotypes are being investigated by Brown, Payne, and Woloshuk. Discoveries made in their studies should provide markers that will assist the conventional breeding effort. Brown compared extractable proteins from resistant and susceptible genotypes by SDS-PAGE. His group found that resistant genotypes produce greater amounts of a 14 kDa trypsin inhibitor than susceptible genotypes. The purified protein was also inhibitory to the growth of several fungi including *A. flavus* and *A. parasiticus*. Preliminary data suggest that the protein may be a mutifunctional proteinase/amylase inhibitor. Such inhibitors have been shown to inhibit insect gut enzymes. Payne has identified inhibitory activities in protein extracts from the resistant corn inbred Tex6. These activities were separated into two fractions; one inhibiting fungal growth and aflatoxin production, and the other inhibiting only aflatoxin production. Two proteins in the growth-inhibiting fraction were found to be similar to zeamatin (osmotin) and chitinase. Both zeamatin and chitinase are known inhibitors of fungi. However, Payne has suggested that the activity against *A. flavus* may indicate that the chitinase is more active in resistant genotypes or that the enzyme works in synergy with zeamatin. Woloshuk has investigated factors affecting susceptibility to aflatoxin production in corn. Alpha-amylase produced by *A. flavus* may

generate aflatoxin-inducing sugars from starch. He described a mutant *A. flavus* strain having a disrupted alpha-amylase gene which produced no detectable alpha-amylase or aflatoxin.

SUMMARY OF PANEL DISCUSSION: The initial discussion started with a question from the audience about the correlation between growth of *A. flavus* and aflatoxin production. Evidence presented by Brown and Payne indicated that inhibitors of growth in maize kernels correlate well with reduced fungal growth and lower aflatoxin production. At the same time there is evidence that growth and aflatoxin production can be separated. Examples noted were the protein fraction described by Payne which inhibits aflatoxin but not growth and a peanut genotype, VAR 27, that supports *A. flavus* growth but low levels of aflatoxin.

Someone asked if trypsin inhibitor which was close to 20% of the protein in some corn lines, was cause for concern regarding animal/human consumption. Zhiyuan Chen answered this from the audience saying that extractable protein comprises only about 10% of total protein in seed, and protein accounts for only 5-10% of the weight of seed. Thus the trypsin inhibitor, though seemingly in high amounts in the electrophoresis gel, is actually in very small amounts, and of no danger when consumed.

There was a question about the specificity of amylase inhibitors. Woloshuk indicated that plant inhibitors are fairly specific. Evidence indicates that these inhibitors have little effect on plant amylases, and they can selectively inhibit amylases from specific insect species.

It was asked if the pedicel region was a weak point of the corn kernel and thus may provide an avenue of infection for the fungus after harvest, and if this was true could the problem be solved by a breeding program to strengthen this tissue. It was noted that the focus of this group has been on preharvest infection, not postharvest infection. Infection across the chalazal region of the kernel has been reported for Fusarium, but studies in Wicklow's lab indicate that this is not a major avenue for preharvest infection by *A. flavus*. Wicklow stressed that work from his lab indicated that splits in the seed coat were an important factor in the preharvest infection process. Brown also stated that he has observed a significant growth of the fungus from the pedicel area.

Nancy Keller asked if there was evidence that different *A. flavus* isolates responded differently on different host genotypes. More pointedly, she asked if there is a need for everyone to use the same strains. Comments from Wicklow, Payne and Wilson indicated that there are differences between strains and isolates but the significance of these differences are not known.

Finally, it was asked by Holbrook, why is there no effort to breeding for resistance in cotton? Peter Cotty indicated that industry representatives and himself discussed the breeding alternative early in the aflatoxin elimination program. Cotton is produced primarily for the lint. The seed is worth only around 15% of the crop's value and as such, lint quality and yield drive breeding programs. Using traditional breeding methods, aflatoxin resistant cultivars may be continually trying to catch up with those bred solely for lint characteristics. It was felt that until commercially useful resistance is identified on other crops and it's nature is known, cotton would proceed without a traditional breeding program for aflatoxin control. However, cotton maintained a strong program directed at developing cultivar resistance through genetic engineering.

PLATFORM PRESENTATIONS

DETERMINATION OF MAIZE KERNEL BIOCHEMICAL RESISTANCE TO AFLATOXIN ELABORATION: MECHANISMS AND BIOTECHNOLOGICAL TOOLS

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It was determined that growth of *Aspergillus parasiticus* in kernels of resistant, moderately susceptible, and highly susceptible maize genotypes was related to aflatoxin production. The fungal strain employed in this study had been transformed with a green fluorescent protein (GFP) reporter system using a constitutive promoter (for monitoring fungal growth). Similar results have been obtained with these three genotypes employing an *Aspergillus flavus* GUS transformant. Using the GFP reporter system requires no other intrinsic or extrinsic proteins, substrates, or cofactors. Also, fluorescence is stable and species-independent, expression can be monitored in living systems, and GFP is inexpensive to use. GFP appears to be a useful tool for monitoring fungal invasion (visually or quantitatively) of maize kernel tissues. *A. flavus* strains have also successfully been transformed with GFP reporter constructs.

Maize kernel proteins in resistant and susceptible genotypes have been screened using SDS-PAGE and investigated for associations with resistance to *A. flavus* infection/aflatoxin production. Both zeamatin and ribosome inactivating protein (RIP), expressed at similar levels in resistant and susceptible kernels, have been identified and found to inhibit *in vitro* growth of *A. flavus*. A 14 kDa trypsin inhibitor was identified and determined to be expressed in high quantities in several resistant genotypes tested and absent or in low quantities in susceptible ones. Bioassays testing the trypsin inhibitor against *A flavus* germinated or ungerminated conidia found it to be highly inhibitory to germination or normal hyphal extension and at low concentrations (38 ug/ml). Frequently, conidia were observed lysed even after short incubation periods with the protein. The trypsin inhibitor also inhibited the growth (at various concentrations) of *A. parasiticus*, *A. niger*, *Fusarium graminearum*, *F. moniliforme*, *Rhizopus stolonifer*, *Penicillium chrysogenum*, *Cercospora kikuchii*, and *Trichoderma viride*. When a bacterial amylase was added to *A. flavus* conidia in PDA medium along with the 14 kDa trypsin inhibitor, no inhibition of germination was observed. Literature describes some trypsin inhibitors as multifunctional, including inhibitory to insect amylase. Further investigation is needed to determine if the 14 kDa protein falls into this category.

The 14 kDa trypsin inhibitor has been mapped using RFLP analysis and found to be located on chromosome 2 in maize. Also, only one copy was found indicating that this is probably the active gene. The existence of other resistance genes in the same region as the trypsin inhibitor gene has not been described in the literature.

Future research will concentrate on characterizing the mode of action of the trypsin inhibitor, screening other maize germplasm for expression of this protein, and for the existence of other resistance-associated proteins. Also, a QTL analysis of the 14 kDa region of chromosome 2 will

be done to determine the percent contribution of this protein to resistance to aflatoxin accumulation, and hopefully to determine gene regulated.

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EVALUATION OF MUTANT B73 AND A632 INBREDS OF CORN FOR RESISTANCE TO AFLATOXIN SYNTHESIS

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A total of 8348 M3 families of mutagenized B73 and A632 inbred lines of maize were previously bioassayed for inhibitors of aflatoxin synthesis in hexane extracts of maize seed. Inhibition of aflatoxin synthesis was a rare phenomenon and only six kernels were found. The assay was a destructive test, but through a process of selfing plants grown from residual seed from the same family ear, the frequency of the trait in subsequent families has been concentrated trait. In 1997, 20 M6 families of A632 and 40 M6 families of B73 were selected for definitive field trials based on agronomic traits and the fact that each family had at least 70% seeds possessing high levels of inhibition of aflatoxin synthesis. Although extracts from the dry seeds may inhibit aflatoxin synthesis, the materials needed to be tested for inhibition of aflatoxin synthesis in the field. Ears on the plants in the field were inoculated twice at the soft dough stage with *Aspergillus flavus* by injection with a syringe and a 14 gauge Hamilton No. 5 point style needle directed through the developing kernels and along the cob. The infected ears were harvested only one week prior to this report, and have not been scored for *A. flavus* infection or assayed for amount of aflatoxin produced.

Members of some mutant families were crossed with Mo17 and the hybrids were planted in a replicated field trial to assess some agronomic traits and yield in comparison with B73 x Mo17 and A632 x Mo17 with non-mutangenized parents. Tassel branch number, plant height, ear height, and grain yield did not differ significantly ($p = 0.05$) among the entries evaluated. Hybrids produced in 1997 were crosses with B100, a current elite inbred line.

The elite mutagenized B73 and A632 germplasm being used in this research was developed originally by Allen D. Wright while he was associated with the USDA-ARS at Iowa State University.

INHERITANCE OF MOLECULAR MARKERS ASSOCIATED WITH, AND BREEDING FOR, RESISTANCE TO ASPERGILLUS EAR ROT AND AFLATOXIN PRODUCTION IN CORN USING TEX6

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Research at the University of Illinois is divided into four interrelated components including: 1) identifying sources of resistance; 2) determining the inheritance of resistance; 3) molecular marker mapping of genes for resistance; and 4) crossing resistance into B73 and/or Mo17 related inbreds.

Identification of Resistance. We have screened more than 1,200 corn inbreds as F1 crosses with susceptible inbreds Mo17 and/or B73 for resistance to Aspergillus ear rot and aflatoxin production. All screening has been done using artificial inoculation. We have identified 13 inbreds that are highly resistant in F1 combinations and resistant as inbreds per se. Our best source of resistance is the inbred line Tex6 which was selfed from a white corn population (PI 401762)that had been grown in the southern U.S. This inbred also has been identified as resistant in studies by others and may have unique proteins that account for part of the disease resistance.

Inheritance of Resistance in Tex6. Inheritance of resistance in the inbred Tex6 has been studied in crosses with susceptible inbreds B73 and Mo17 following inoculation in the field. From 1994 to 1996 plant generations included were the susceptible parent (P_1), the resistant parent (P_2), F_1, F_2, F_3, BCP_1, BCP_1-selfed, and BCP_2. The BCP_2-selfed generation was added in 1995-1996 for the B73 X Tex6 cross. In general, F_1 means deviated from the mid-parent value for resistance to aflatoxin production in both crosses indicating dominance for resistance. Analysis of generation means indicate that additive gene action is of primary importance for resistance to aflatoxin production in both crosses. Broad sense heritabilities for aflatoxin resistance were 63% for Mo17 x Tex6 and 65% for B73 x Tex6. Our biggest problem has been the classification of a family as being resistant that is genetically susceptible in years that do not favor aflatoxin production. With these families, susceptibility becomes apparent in subsequent years that favor aflatoxin production.

Mapping genes for Resistance. Genotypic analysis of the Tex6 x Mo17 F_3 mapping population has been completed with approximately 90 RFLP and SSR markers. Because of the variability of phenotypic traits between years, a modified data set was created for aflatoxin. In an attempt to identify those families with consistently low levels of aflatoxin, concentrations were scaled relative to Mo17 then the highest of the two years was used in the analysis. Using this data set, a stepwise multiple regression model from SAS including three probes accounted for 27% of the variation for aflatoxin. Individual year multiple regression models include 7-9 markers explaining approximately 22% of the variation. Three chromosomal regions were found to be significantly associated with resistance in the multiple regression (1L, 2L, 4S). Analysis of 21 "resistant" families indicated that 15 of these families were either homozygous Tex6 or heterozygous at all three regions. Five

were either homozygous Tex6 or heterozygous at two regions. Comparison of the Tex6 x Mo17 F3 population with previous mapping populations (LB31 x B73, 75-R0001 x B73) indicates that while there are common regions associated with aflatoxin resistance, there also are some regions unique to one inbred associated with resistance. For example, all three resistant parents confer resistance on 3L, 4S whereas only Tex6 confers resistance on 3S. We are in the process of mapping the B73 x Tex6 F_3 population and the B73 x Tex6 backcross to B73 self population. If regions associated with resistance in these populations are similar to the Mo17 x Tex6 F_3 population this will indicate that we have identified chromosomal regions associated with resistance that are not in either B73 or Mo17. Genes in these regions would be very valuable in most cornbelt germplasm.

Breeding for disease resistance. We've made progress in transferring resistance from the inbred LB31 into B73 related inbreds while maintaining yield. We've also been able to backcross resistance from Tex6 into both B73 and Mo17 related inbreds. Our ultimate goal is to have B73 x Mo17 type hybrids with resistance in both parents. This should result in a hybrid that could be used in areas where aflatoxin is a problem in most years. Highly resistant hybrids may need to have genes pyramided from different sources of resistance.

Conclusions. High levels of resistance have been identified and can be transferred into usable germplasm. In most cases, it appears that resistance is under the control of several genes acting in an additive fashion; however, these genes confer resistance in F_1 hybrids. Marker assisted selection is not a reality at this time, however, it has provided valuable information on chromosomal regions associated with resistance from different sources of resistance. It seems reasonable that since different sources of resistance have some chromosomal regions associated with resistance that are different that they may have some different genes for resistance. Mapping populations are starting to provide information that would allow us to pyramid resistance genes from different resistant parents. Also, we will be able to recombine lines during backcrossing to produce backcrossed derived lines with all of the chromosomal regions associated with resistance. The B73 x Tex6 and Mo17 x Tex6 mapping population should provide information about resistance genes not found in most cornbelt germplasm.

AFLATOXIN AND BRIGHT GREENISH-YELLOW FLUORESCENT KERNELS IN A COMMERCIAL CORN HYBRID INOCULATED WITH *ASPERGILLUS FLAVUS* GENOTYPES ISOLATED FROM CORN IN ILLINOIS

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Nineteen strains of *Aspergillus flavus*, including 15 genotypes (DNA fingerprinting), isolated from corn grown near Kilbourne, IL were evaluated for their ability to produce bright greenish-yellow fluorescent (BGYF) kernels and aflatoxins in a commercial corn hybrid (Pioneer 3394) grown at the same location. Strain selection was guided by an evaluation of aflatoxin-producing ability (13 positive; 6 negative) in yeast extract soytone broth as determined by TLC. Tooth-pick wound-inoculations were performed with each *A. flavus* strain on ten or more corn ears in the late milk to early dough stage of maturity (21 days following mid-silk). At harvest, the 20 kernels nearest each wound-site were segregated into three categories: wound-inoculated kernels, non-wounded BGYF kernels, all other non-wounded kernels. Sample weights of wound-inoculated kernels averaged 0.6% (range= 0.4% - 3%) while sample weights of BGYF kernels averaged 5% (range = 1.5% - 11.5%) of the total sample weight. Aflatoxin levels among eleven samples of wound-inoculated kernels varied sixteen fold (mean = 10,500 ppb; range = 1,060 ppb-17,200 ppb) while related samples of non-wounded BGYF kernels varied seventy-three fold (mean = 1,100 ppb; range = 52 ppb - 3,800 ppb). Greater sensitivity to kernel resistance factors may explain why some *A. flavus* genotypes (e.g., GT#02, NRRL 27676; GT#37 NRRL 26477, etc.) showed a proportionately greater suppression in aflatoxin following infection of the germ and endosperm. Removal of both the aflatoxin- contaminated wound-inoculated kernels and non-wounded BGYF kernels afforded grain samples with a mean aflatoxin value of 2 ppb (range = ND - 14 ppb).

RESULTS FROM SCREENING THE PEANUT CORE COLLECTION FOR RESISTANCE TO PREHARVEST AFLATOXIN CONTAMINATION

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Preharvest aflatoxin contamination (PAC) is one of the most serious challenges facing the U.S. peanut industry. The objectives of this research program are to identify sources of resistance to PAC and to use these sources to develop resistant peanut cultivars. To facilitate the identification of resistant genes, a core collection was selected to represent the entire germplasm collection for peanut. All available data for accessions in the entire germplasm collection (7,400 accession) were used to cluster the accessions into genetically similar groups. Random sampling was then used to select ten percent from each group. The resulting 831 genotypes form the core collection for peanut. All accession in the core collection were first examined in a preliminary screen using five replications in a single environment. Genotypes that had low aflatoxin contamination levels in the preliminary screen were then retested. Fifteen core accessions (47, 66, 99, 147, 158, 174, 215, 276, 282, 287, 292, 299, 511, 522, 554) have showed low levels of aflatoxin contamination in multiple environments. These accession have exhibited a 70 to 90 % reduction in aflatoxin contamination in comparison to susceptible accessions in multiple environments. The lines that we have identified as having lower PAC have less than acceptable agronomic characteristics. These lines have been entered into a hybridization program to combine resistance to PAC with acceptable agronomic performance. We are also intermating the resistance lines. The objective of this is to combine different genes for resistance to produce genotypes with even higher resistance to PAC.

CHARACTERIZATION AND FIELD TESTING OF AN INTEGRATED FUNGAL PATHOGEN/INSECT VECTOR RESISTANCE TO AFLATOXIN CONTAMINATION OF ALMOND

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Previous research has shown that the critical period for preharvest aflatoxin contamination occurs during the limited period from hull split to the field drying of almond kernel meats. Aflatoxin contamination occurs when the seed coat, which normally acts as a barrier to infection by *Aspergillus flavus* (AF) is damaged by Navel orangeworm (NOW) *(Amyelois transitella)* feeding. An integrated resistance to preharvest aflatoxin contamination is thus being pursued through the development of resistance to NOW in the form of hull and kernel antibiosis/nonpreference and/or endocarp (shell) seal integrity, combined with resistance in the kernel and seed coat to AF. Specific goals for 1997 include the improved characterization of these resistance mechanisms including their performance in regional field trials, the development of breeding lines integrating multiple resistances, and the development of more efficient transformation and regeneration methods for commercially important cultivars demonstrating recalcitrance to the necessary adventitious regeneration.

Multi-year feeding trials involving both intact hulls and nuts, as well as controlled concentrations of hull and kernel tissue incorporated into standard NOW diets have now been completed for important almond cultivars. Statistically significant differences have been identified for certain genotypes, including the historically important cultivar 'Mission'. Considerable variation between replications was observed even when controlled diets and environments were employed. The identification of the specific compounds associated with resistant genotypes are thus being pursued in cooperation with USDA, Albany, CA researchers since chemical assays should be a more objective and efficient selection index then the more erratic insect growth studies.

Genotypes selected for their endocarp seal integrity as characterized by X-Ray analysis, seal integrity in pressurized systems, and by visual (anatomical) analysis were found to possess both an effective and durable barrier to NOW infestation for the range of regional field sites evaluated. Almost all worm infestation of resistant genotypes was due to NOW feeding with a few cases of Peach Twig Borer (PTB) *(Anarsia lineatella)* infestation. A thin yet well sealed and durable endocarp barrier has now been transferred from selected parents to progeny, though the genetic control of this trait has not yet been determined.

Kernel fatty-acid composition for important cultivars and breeding lines has now been characterized with significant differences identified. Although initial analysis has not detected any significant associations between kernel composition and either NOW or AF resistance, certain high oleic/linoleic genotypes which resulted from interspecific gene introgression have been shown by Dr. N. Mahoney, USDA, Albany, CA to result in dramatically lower levels of aflatoxin formation following controlled inoculations and culture.

Although the cultivar 'Nonpareil' has now been successfully transformed, regeneration methods based on successfully developed protocols for Almond x Peach hybrids have failed for this almond cultivar. More genotype specific protocols are now being pursued along with the capacity for the direct particle bombardment of inherently regenerable shoot meristems.

CHARACTERIZATION OF INHIBITORS FROM CORN SEEDS AND THE USE OF A NEW REPORTER CONSTRUCT TO SELECT CORN GENOTYPES RESISTANT TO AFLATOXIN ACCUMULATION

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Research in my lab has centered in five areas: 1) overexpression of the pathway regulatory gene, *aflR*; 2) characterization of *aflJ*; 3) characterization of the DNA binding site in the nor-1 promoter; 4) development of three reporter gene constructs; and 5) characterization of a compound from corn inbred Tex6 inhibitory to *A. flavus*. To examine the effect of *aflR* overexpression on temporal gene expression, aflatoxin production, and nitrate inhibition of aflatoxin biosynthesis in *A. flavus*, the constitutive promoter *gpdA* was fused to *aflR* (5). Transformants with this construct constitutively expressed transcripts for *aflR*, *fas-la*, *pksA*, *nor-1* and *omtA*, but did not constitutively produce aflatoxin. Strain 86-10 containing the *gpdA(p)::aflR* construct produced 50 times more aflatoxin than 86-10, but the temporal pattern of aflatoxin production was the same as for 86-10, and aflatoxin production was also induced by sucrose. The addition of 10 g of nitrate per liter to sucrose low salts medium inhibited aflatoxin production by both strain 86-10 and a transformant of 86-10 containing the *gpdA(p)::aflR* construct, indicating that nitrate inhibition of aflatoxin biosynthesis does not occur solely at the level of transcription. These studies show that over expression of *aflR* leads to higher levels of aflatoxin production but that the initiation of aflatoxin biosynthesis is not solely regulated by the transcriptional activities of the biosynthetic pathway. Thus, some other regulatory mechanism must be involved.

We have been investigating *aflJ* and its possible role in the regulation of aflatoxin biosynthesis. This gene resides in the cluster adjacent to the pathway regulatory gene, *aflR*, and the two genes are divergently transcribed. Disruption of *aflJ* in *A. flavus* results in its failure to convert the pathway intermediates norsolorinic acid, sterigmatocystin, and O-methyl sterigmatocystin to aflatoxin. The disrupted strain does however, accumulate transcripts of *pksA*, *nor-1*, *ver*-1, and *omtA* under conditions conducive to aflatoxin biosynthesis. Thus, disruption of *aflJ* prevents aflatoxin accumulation, but does not affect transcription of the pathway genes. Sequence analysis of *aflJ* and the putative peptide, AflJ, did not reveal any enzymatic motifs or significant similarities to proteins of known function. The putative peptide does contain three regions predicted to be membrane spanning domains and has a AAA signature. Whatever the function of *aflJ*, the gene is a good target site for inhibition as there are no pathway conversions when this gene is disrupted.

In order to better understand the regulation of aflatoxin biosynthesis by the pathway specific regulatory gene, the binding of *aflR* to the *nor-1* structural gene promoter has been investigated by electrophoretic mobility shift assays and deletion analysis of the *nor-1* promoter fused to the GUS reporter gene. We have identified a putative binding site for *aflR* in the region spanning -103 to -88 of the 5'-UTR of the *nor-1* promoter.

To facilitate the monitoring of aflatoxin pathway gene expression and fungal growth, we have developed three new reporter constructs. We have previously shown the utility of using reporter genes to measure growth and aflatoxin production by *A. flavus* (2,6). The construct *omtA*(p)::GUS was made by fusing the promoter of the pathway gene *omtA* to the β-glucuronidase (GUS) reporter gene. We showed that expression of this construct in *A. flavus* follows the same profile as aflatoxin biosynthesis. We also engineered two reporter

constructs based on the green fluorescent protein (gfp) from *Aequorea victoria* (3,4). This reporter has several advantages over GUS reporters. First, the fungus can be visualized in live tissue without fixation or addition of specific substrates, thus making screening faster and more economical. Secondly, because GFP is a vital reporter, it is possible to monitor the growth of the fungus in living tissue. Finally, the use of GFP reporter constructs does not require sophisticated equipment for the monitoring of growth and colonization. The first gfp construct was made by fusing the promoter of the cytomegalo virus, *cmv*, to gfp. This construct, cmv(p)::gfp, will be useful in studies designed to monitor fungal growth. The second reporter construct was made by fusing the promoter and 483 bp of the open reading frame from the aflatoxin pathway specific regulatory gene, *aflR* to gfp. Expression of gfp by this construct follows the same profile as aflatoxin accumulation in infected corn kernels, thus, this construct will be useful in screening corn genotypes for resistance to aflatoxin accumulation.

The final area of research is on the inhibitory protein from corn inbred Tex6. Seed of Tex6 have been shown to have resistance to aflatoxin contamination (7). We have identified two inhibitory activities in Tex6, one that inhibits fungal growth and the other that inhibits aflatoxin biosynthesis(7). We have focused on the growth inhibitory compound. In a partially purified extract, the inhibitory activity is associated with two bands on an SDS-PAGE gel. The faster migrating band reacts with antibodies of zeamatin and thus appears to be zeamatin or a closely related compound. Partial peptide sequence of the slower migrating band indicates that it is a chitinase. It shows similarity to a known chitinase from corn. At this point, we have not determined if it is a chitinase that has been reported or an unidentified chitinase with similar structure. Two chitinase have been reported from corn with 85% sequence similarity that have very different biological activity, thus the chitinase we have identified may be one with much higher antifungal activity than others that have been found. We also cannot rule out the possibility that the chitinase is acting in conjunction with another compound to inhibit growth of *A. flavus*. Our focus now is to purify the compound and make antibodies to it for use in screening breeding populations for the presence of the compound. We will also clone the gene coding for the chitinase and use it as a probe in marker assisted breeding.

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AFLATOXIN-INDUCING METABOLITES IN MAIZE KERNELS: ARE THEY POTENTIAL TARGETS FOR AFLATOXIN ELIMINATION?

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Research effort in my laboratory has focused on the identification of metabolites in corn kernels that are involved in the induction of aflatoxin biosynthesis. These metabolites can be considered susceptibility factors that are inherently part of the interaction between *Aspergillus flavus* and corn. Altering the production or the availability of these metabolites through methodologies such as breeding, chemicals, bioengineering or biocontrol would ultimately lower aflatoxin production.

A reporter-gene assay was developed that could readily measure molecular activation of aflatoxin biosynthesis. The promoter of one pathway gene (*ver1*) was cloned immediately upstream of the ß-glucuronidase (GUS) gene (*uidA*) from *E. coli*. Data indicated that the *ver1*:GUS construct in an *A. flavus* transformant (GAP13-22) could be utilized effectively to examine the timing and magnitude of gene expression of at least one gene in the aflatoxin biosynthetic pathway.

Using the GUS reporter assay, an aflatoxin-inducing activity was detected in culture filtrates of the aflatoxigenic *A. flavus* strain NRRL 3357 grown on maize kernels. The inducing activity passed through ultrafiltration membranes with 10 kDa exclusion and was not inactivated by autoclaving for 15 minutes. Data suggested that the inducing activity was the degradation products of a complex seed molecule. Further characterization the aflatoxin-inducing activity indicated the presence of glucose, maltose, and maltotriose in near equal molar concentrations (about 15 mM). We determined that the minimum concentration of glucose, maltose or maltotriose that induced measurable GUS activity was 1 mM. This concentration was 10 times less than previous studies indicating that a mechanism regulating aflatoxin biosynthesis is activated at low levels of carbon. From these data, we hypothesized that the complex molecule was starch and that an amylase produced by *A. flavus* degraded the starch to aflatoxin-inducing glucose, maltose, and maltotriose. *A. flavus* produces a single α-amylase with a pI of 4.3. No maltase or amyloglucosidase has been detected.

This year we are trying to determine if α-amylase produced by *A. flavus* has a role in the induction of aflatoxin biosynthesis in infected maize kernels. We disrupted the α-amylase gene *amyA* in an aflatoxin-producing strain of *A. flavus*. The characterization of this mutant is presented as a poster by Fakhoury and Woloshuk. We are currently determining to what extent this α-amylase-deficient mutant colonizes corn kernels and produces aflatoxin.

STUDIES ON THE IDENTIFICATION OF RESISTANCE TO AFLATOXIN CONTAMINATION OF CORN USING AN *ASPERGILLUS PARASITICUS* MUTANT

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The norsolorinic acid producing (nor) *Aspergillus parasiticus* mutant has the aflatoxin pathway partially blocked. This mutant produces nor in corn kernels as well as aflatoxin and thus is useful for visual observations. The initial studies were to determine if the pigment could be used to identify highly susceptible genotypes. The subsequent studies were to evaluate the mutant and wild type *A. flavus* isolates in studies on invasion of the ear.

1. The mutant was shown to be useful in separating highly susceptible genotypes from those which might be resistant to aflatoxin contamination.

2. In wounded ears, there was a negative correlation between the mutant and the wild type *A. flavus*. However silk inoculation by the mutant was not effective.

3. The percentage of red kernels observed varied by year and seemed to be related to weather conditions.

4. The occurrence of red kernels was less in years with high wild type *A. flavus* populations in the ear.

5. The recovery of the mutant was not related to the wound site. The mutant successfully colonized the ear whenever the inoculum was introduced by wounding.

The studies have been useful in describing the distribution of the fungi on the ear and the mutant could easily be used to help in a screening program.

POSTER PRESENTATIONS

ASPERGILLUS FLAVUS GROWTH AND AFLATOXIN ACCUMULATION IN 15 MAIZE ISOLINES CONTAINING GENES FOR ANTHOCYANIN PRODUCTION

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Aspergillus flavus infects numerous agronomically important crops. In addition, it can produce a carcinogenic compound, aflatoxin. Previous research indicates that compounds produced by the anthocyanin pathway, which controls pigment production in the kernel and other plant parts, may reduce the growth of *A. flavus* and/or the production of aflatoxin. This pathway is biochemically and genetically well characterized. Four replicates of 15 maize lines each containing a different gene or combination of genes involved in kernel pigmentation or starch biosynthesis were infected with *A. flavus* isolate NRRL 3357. Mature kernels were plated to determine percent infection and aflatoxin levels measured. The line containing *in1*, which produces an intense purple kernel had reduced fungal infection compared to the control, a line containing the alleles present in commercial maize lines. It did not have reduced aflatoxin levels. Lines containing genes such as *sh, su*, or *bt* which increase the proportion of simple sugar in the endosperm had the highest infection rates and aflatoxin levels. In addition, lines containing the *c2* and *c2-I df* alleles had significantly higher aflatoxin levels without increasing fungal infection. These results support the hypothesis that toxin production and fungal growth are affected by different genes in the maize plant. Further studies are needed to determine the proportion of variation in resistance/susceptibility to *A. flavus* that is controlled by these genes in breeding materials.

ANTIFUNGAL PROTEINS IN CORN KERNELS: IMMUNOCHEMICAL LOCALIZATION AND INDUCTION DURING GERMINATION

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This study examined antifungal protein, RIP and zeamatin, distribution and localization in kernel tissues and induction during imbibition and germination as well as *in vitro* bioassay of purified protein and extracts from dry or germinating kernels against *Aspergillus flavus* and *Fusarium moniliforme*. Vertical and cross-sectional kernels were blotted onto nitrocellulose. Antibody against maize RIP (ribosome-inactivating-protein) and zeamatin were used for immunochemical localization of the protein in different kernel tissues. RIP was found primarily in aleurone layer of endosperm tissue and zeamatin was located mainly in kernel embryo. Control using preimmune rabbit serum did not give positive reaction. Protein extractions were separated by SDS-PAGE and revealed by western blot analyses. One protein with 22 kDa molecular mass reacted with zeamatin antiserum and accumulated to a higher concentration in germinated kernels. Two protein bands from dry kernels which reacted with RIP antiserum were identified as the 32 kDa proRIP-like form and one 18 kDa peptide. However, in germinated kernels, two protein bands which reacted with RIP antiserum were identified as two active RIP-like peptides of molecular mass about 18 kDa and 9 kDa, respectively. Purified RIP and zeamatin from corn inhibited growth of *A. flavus*. Bioassays of germinated kernel extracts exhibited stronger antifungal activity against *A. flavus* and *F. moniliforme* than extracts from dry kernels, especially extracts from the susceptible genotypes showing greater inhibition zones. This study indicates that the localization of RIP and zeamatin in different kernel tissues may be related to fungal infection pathway and also provides evidence of protein induction in corn kernels during imbibition or the early stages of germination to support the germination-associated resistance in the corn kernel.

EXPRESSION OF THE GREEN FLUORESCENT PROTEIN IN *ASPERGILLUS FLAVUS* AND ITS USE AS A MARKER TO EVALUATE RESISTANCE IN CORN KERNELS

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Aflatoxins are extremely carcinogenic and toxic secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. We report here the development of two reporter gene constructs based on the jellyfish green fluorescent protein, GFP, that will allow us to study the growth of *A. flavus* and aflatoxin biosynthesis. The two expression constructs (1,2) were made in frame with the entire coding region of gfp from *Aequorea victoria*. One construct contains the cytomegalo virus promoter, *cmv*, and the other one contains the promoter and 483 bp of the open reading frame from the aflatoxin pathway specific regulatory gene, *aflR*. The expression of GFP in the transformants harboring the *cmv::gfp* construct was sufficient to monitor fungal growth and to allow visualization in corn kernels infected with the fungus. Transformants harboring the *aflR::gfp* construct temporally expressed GFP with a profile consistent with that previously found for GUS reporter constructs (3). The expression of GFP in these transformants was evaluated by inoculating different corn genotypes. In both susceptible and resistant kernels, GFP expression was correlated with aflatoxin B_1 production. Expression of GFP differed between the two constructs and was influenced by kernel genotypes. These transformants should provide a tool for monitoring *A. flavus* growth and aflatoxin contamination in corn kernels in the field. Further, these constructs will allow us to monitor *aflR* gene expression and traffic the localization of AFLR *in vivo*.

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CONSTRUCTION AND ANALYSIS OF AN *ASPERGILLUS FLAVUS* OMTA (P)::GUS REPORTER CONSTRUCT

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Aflatoxins are the most carcinogenic naturally occurring compounds known. These secondary metabolites are produced on such crops as corn, peanuts, cottonseed, and tree nuts. Effective control strategies are not available to eliminate aflatoxin accumulation. To better understand the molecular biology of aflatoxin biosynthesis, we are developing and utilizing both genetic and molecular tools. One powerful tool for understanding the factors that affect growth and aflatoxin production by *A. flavus* is a gene reporter system (1,2,3). We previously showed that a reporter construct containing the promoter of the *A. flavus* B-tubulin gene fused to the E. coli β-glucuronidase gene (GUS) is a reliable tool to measure fungal growth in corn kernels (4). Both laboratory and field studies have shown that colonization of corn kernels by *A. flavus* can be monitored easily and accurately by GUS expression. Here we report the construction and analysis of a new reporter gene construct utilizing the *omtA* gene promoter of the aflatoxin biosynthetic pathway. Expression of GUS activity by this construct (*omtA*(p)::GUS) parallels that of aflatoxin accumulation in culture. In transformant GAP26-1 harboring this construct, aflatoxin production and GUS expression on sucrose-containing medium show the same temporal pattern of induction. We further showed that GUS expression and aflatoxin accumulation are correlated in corn kernels inoculated with GAP26-1. Thus this construct should prove to be a valuable tool to monitor the expression of an aflatoxin pathway gene and to evaluate corn genotypes for their resistance to aflatoxin accumulation.

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MICROBIAL ECOLOGY

PANEL DISCUSSION

PANEL DISCUSSION TITLE: Microbial Ecology

PANEL MEMBERS: Joe Dorner (Chair), Peter Cotty, Bruce Horn, Merritt Nelson, Don Wicklow and Gary Windham

SUMMARY OF PRESENTATIONS: Peter Cotty discussed results of studies conducted under an Experimental Use Permit in Arizona on the use of wheat colonized by *Aspergillus flavus* AF36 for biological control of aflatoxin contamination of cottonseed. Three fields (120 acres) were treated at 10 lb per acre in June, 1996, and soil analysis prior to application showed that AF36 comprised a low percentage of the resident *A. flavus* community. At harvest, however, AF36 was the dominant *A. flavus* strain contaminating seed surfaces. Data also showed that the incidence of AF36 increased on crops produced in adjacent, untreated fields. The incidence of AF36 remained high in soil in 1997, even in a field that had undergone crop rotation, indicating the potential for a long-term influence with applications of AF36.

Merritt Nelson and coworkers have studied the composition of *A. flavus* isolates with regard to the S and L strains in Yuma County, Arizona, for several years. Strain composition of soil appears to be remarkably consistent over time and does not correspond with the crop or crop sequence. Of particular interest is the incidence of the S strain because it has been shown consistently to produce large amounts of aflatoxin. Efforts to map the distribution of the S strain have been aided using a geostatistical approach, and analysis of 1997 data revealed that S strain incidence over much of the Texas Hill area averaged above 60%. Successful mapping of areas high in S strain incidence should help focus attention on those areas with the greatest potential for aflatoxin problems.

Bruce Horn reported on soil populations of *Aspergillus* species from section *Flavi* found along a transect from eastern New Mexico to Georgia then northeast to Virginia. Soil was sampled primarily from peanut fields in the four major peanut-growing regions and also from corn, cotton, and soybean fields from other regions. *A. flavus* was the most dominant species from section *Flavi* found, but peanut fields had lower populations of *A. flavus* compared with other crops. Soil populations of *A. parasiticus* were significantly higher in peanut fields compared with other crops. Analysis of S and L strains of *A. flavus* for production of aflatoxin and cyclopiazonic acid (CPA)showed that the S strains produced higher concentrations of aflatoxin, the L strains were more variable in their ability to produce toxins, and nearly all S strains produced both aflatoxin and CPA.

Joe Dorner discussed the potential for biological control of preharvest aflatoxin contamination of corn by applying nontoxigenic strains of *A. flavus* and *A. parasiticus* to soil. After application of strains to soil in Southwest Georgia test plots between 1994 and 1997, significant reductions in contamination of corn were seen in 1996 and 1997. This coincided with reduced colonization of corn by wild-type *A. flavus* in treated plots compared with untreated plots. However, there was no correlation between fungal soil populations and fungal colonization of corn in either 1996 or 1997. Data indicated that the reduction in aflatoxin contamination seen in treated corn resulted primarily from the addition of the nontoxigenic *A. flavus*; the inclusion of the nontoxigenic strain of *A. parasiticus* did not seem to play a role in reducing aflatoxin.

SUMMARY OF PANEL DISCUSSION: The discussion began with Gary Windham explaining their use of an *A. flavus* isolate containing a GUS reporter gene to study colonization of corn. After inoculating ears, GUS activity was found in the embryo as well as in the vascular tissue of the cob under infected kernels. Use of such modified strains of *A. flavus* should add greatly to the understanding of the colonization and infection of corn by *A. flavus*.

Much of the panel discussion centered on the relationships of species in section *Flavi* and their association with various crops. Reference was made to the presentation by Horn concerning the identification of species in section *Flavi* that he found along a transact through peanut-growing regions of the United States. He concluded that although he did not know the cropping history of the fields he sampled, he thought that the history of crops grown in a geographic region would have an influence on soil populations. He confirmed that *A. parasiticus* was associated more with peanut fields than with fields containing corn, cotton, or soybeans. He was asked to describe *A. caelatus*, a newly described species in section *Flavi*. He noted that it was morphologically similar to *A. tamarii*, but that the colony color of *A. caelatus* was greener. It has a texture that is similar to *A. parasiticus*, and its spores are highly tuberculate. It produces a diffusible yellow-brown pigment when grown on Czapek agar medium.

There was considerable interest in the distribution of S and L strains of *A. flavus*. Part of Merritt Nelson's research has focused on correlating occurrence of the S strain with certain soil characteristics. Preliminary analysis showed a negative correlation of the S strain with boron and a positive one with the sand content of soil. He tentatively concluded that sand distribution might be one reasonable explanation for the patterns of S strain distribution he has observed. Wicklow noted that all of the *A. flavus* isolates he obtained from corn ears grown in the Illinois River Valley sand fields were L. strains; however, Cotty has found S strains in corn grown in Arizona. He has also found sclerotium formation by S strains in cotton bolls and seed to be quite common in Arizona. In soil samples gathered along the transect from New Mexico to Virginia by Bruce Horn, the highest incidences of the S strain occurred in east-central Texas and Louisiana, which were areas along the transect that were characterized by intensive cotton cultivation. It is apparent that much more work needs to be done to come to a better understanding of the distribution of S strains of *A. flavus*.

PLATFORM PRESENTATIONS

THE USE OF *ASPERGILLUS FLAVUS* TO PREVENT AFLATOXIN CONTAMINATION IN COMMERCIAL AGRICULTURE

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Applications of steam sterilized wheat colonized by *Aspergillus flavus* AF36 were made to commercial cotton fields in Yuma County, Arizona in accordance with Experimental Use Permit 69224-EUP-1. In 1996 a total of 3 fields (~120 acres) were treated at 10 lb per acre. Both aflatoxin and fungal analyses suggest AF36 successfully displaced aflatoxin producing strains and reduced the quantity of aflatoxins in the crop. Regression analyses indicated that the quantity of aflatoxin in the crop was inversely related to the incidence of *Aspergillus flavus* AF36. Prior to application in June 1996, 104 soil samples were taken from the 3 treated and 13 nearby fields. Approximately 1,350 isolates of *A. flavus* were cultured from these soils and incidence of AF36 was determined by vegetative compatibility analyses. AF36 was 1.2% to 8.6% of the resident *A. flavus* community prior to application and became dominant on the crop after application composing from 74.2% to 98.6% of the *A. flavus* on seed surfaces after ginning. Applications also increased incidence of AF36 on the crops produced in fields adjacent to treated fields. Soil samples (146) were taken in late May and early June from 25 fields in the Yuma and Mohawk Valleys in order to assess second year influences of the 1996 atoxigenic strain applications and to determine incidence of applied strains in fields to be treated initially in 1997. Analyses of the fungi resident in these soils indicate long-term influences of applications on the *A. flavus* community structure in both treated and nearby fields. Incidence of AF36 within the soil *A. flavus* community of treated fields averaged 85.3% one year after applications were made. Long-term influences were even seen in the one treated field rotated out of cotton in 1997. That field was planted to wheat in December seven months after treatment with AF36. After wheat harvest in June 1997 AF36 remained the dominant strain in the soil being 89.7% of all *A. flavus* isolates. The results of the 1996 tests suggest applications of atoxigenic *Aspergillus flavus* strains are compatible with commercial agriculture and may be particularly useful in area-wide programs directed at reducing aflatoxin contamination throughout entire agricultural communities. Changes induced in *A. flavus* communities by applications of AF36 can survive crop rotations. This is vital to long-term success in regions like western Arizona where crops are grown year-round.

Two pesticide label amendments for *Aspergillus flavus* AF36 were obtained from the EPA in 1997. These allow aerial application and a change in packaging (to 5 gallon buckets). Manufacture techniques were scaled up and over 4,500 pounds of material was manufactured and shipped to Arizona by truck and stored in sheds and warehouses without refrigeration for two to six weeks prior to application. Growers applied material to 463 planted acres; 153 with Gandy box applicators, 78 with a fertilizer spreader, and 232 by plane. The material was compatible with all the application methods. Samples of the applied material were returned to the laboratory for vegetative compatibility analysis of the released fungi to ensure the product functioned according to specifications.

POTENTIAL FOR BIOLOGICAL CONTROL OF PREHARVEST AFLATOXIN CONTAMINATION OF CORN USING COMPETITIVE, NONTOXIGENIC STRAINS OF *ASPERGILLUS FLAVUS* AND *A. PARASITICUS*

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Soil in eight corn plots (18 × 80 ft.) was inoculated with nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus* during crop years 1994-1997 to determine the effect of the nontoxigenic strains on aflatoxin contamination. Eight corn plots in a separate part of the field were not inoculated and served as controls.

In 1994-1995, aflatoxin contamination did not occur to any significant degree, probably because environmental conditions in those years were relatively cool and wet. In 1996, the aflatoxin concentration in corn from treated plots averaged 23.6 ppb, a significant reduction ($p < 0.001$) compared with the aflatoxin in control plots, which averaged 188.3 ppb. This coincided with a significantly ($p = 0.018$) reduced colonization of corn by wild-type *A. flavus* in treated plots (1.8% of kernels) compared with control plots (5.4%). However, there was no correlation between soil populations of inoculated and wild-type strains and colonization of corn. In treated plots, soil populations consisted of: inoculated *A. parasiticus*, 1256 CFU/g; inoculated *A. flavus*, 319 CFU/g; and wild-type *A. flavus*, 195 CFU/g. The percentage of kernels colonized by inoculated *A. parasiticus*, inoculated *A. flavus*, and wild-type *A. flavus* averaged 0.8, 3.3, and 1.8%, respectively.

In 1997, aflatoxin was again significantly reduced ($p = 0.024$) in treated corn (29.8 ppb) compared with untreated corn (87.5 ppb). Treated corn was predominately colonized by the introduced strain of *A. flavus* (26.7% of kernels) compared with wild-type *A. flavus* (2.9%) and inoculated *A. parasiticus* (<1.0%). The lack of a correlation between fungal colonization of corn and fungal soil populations that was seen in 1996 was confirmed in 1997, since the inoculated *A. parasiticus* again dominated in the soil with 1725 CFU/g while wild-type and inoculated strains of *A. flavus* totaled only 814 CFU/g. These data indicate that a degree of biological control of aflatoxin contamination of corn can be achieved by inoculating soil with an aggressive, nontoxigenic strain of *A. flavus*. In these studies, inclusion of the nontoxigenic strain of *A. parasiticus* apparently was not a factor in reducing aflatoxin in corn.

GEOGRAPHIC INFORMATION SYSTEMS (GIS) REVEAL RECURRING PATTERNS OF S AND L FORMS OF *ASPERGILLUS FLAVUS*

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The *Aspergillus flavus* community at the soil surface is genetically diverse. The community can be sub-divided into two strains (S and L) based on the morphology and growth rate of the sclerotia. S strain isolates consistently (>98%) produce large amounts of aflatoxin. Since 1994, repeated sampling of soil in Yuma County, AZ cotton growing areas has shown that S strain incidence is patchy and that patches persist over time. Sampling of adjacent fields have demonstrated that patches of low S strain incidence extend beyond field boundaries and do not correspond in an obvious way with the crop in the field or with field crop sequence. Many locations have shown remarkable consistency in strain composition over time. For example, samples from the NE corner of field 1 in the Texas Hill area have had an S strain incidence between 80% and 100% in eight consecutive samplings from April 1995 through July 1996. Samples from the NE corner of field 11, located 3 miles east of field 1, have ranged between 15% and 55% S strain in eight consecutive samples and have been under 35% S strain, seven of the eight times.

Because S strain incidence is relatively consistent over time, strain composition maps of regions will have a significant impact on the application of management strategies. In July 1997, we began sampling from other locations broadly distributed in the Texas Hill and North Gila areas of Yuma County so that we can better map S strain incidence. The point data can be interpolated using geostatistics to produce either grid cell maps or contour maps. Variogram analysis shows strong spatial autocorrelation with a range of 3.5 km. This supports appropriateness of a geostatistical approach to the mapping and is a refinement on our previous estimate of important spatial structure between 1 and 5 km using nested ANOVA's. Using the July 1997 data, we have generated preliminary sub-regional maps of the Texas Hill area. Much of the area averages over 60% S strain with a significant subset averaging above 80% S strain. However, there are three patches where incidence averages below 60% S.

Using geographic information system (GIS) software, we can overlay S strain incidence contours onto USDA SCS soil maps. The patches of low S strain incidence are in different soil types and the highest S strain incidence areas include diverse soil types. Therefore, our analysis of soil characteristics is going beyond the USDA SCS soil classification scheme.

The successful mapping of the distribution of the key S strain, is important in providing a means of focusing attention on those areas with the greatest potential for aflatoxin problems. The rapidly developing "Precision or Site Specific Farming" technology will provide a new tool for efficient utilization of spatial data in the application of management programs on a regional scale.

SOIL POPULATIONS OF *ASPERGILLUS* SPECIES FROM SECTION *FLAVI* ALONG A TRANSECT THROUGH PEANUT-GROWING REGIONS OF THE UNITED STATES

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Soil populations of *Aspergillus* species from section *Flavi* were examined in 166 fields along a transect that extended from eastern New Mexico to Georgia then northeast to Virginia. Fields were from four major peanut-growing regions (western Texas, central Texas, Georgia/Alabama, and Virginia/North Carolina); corn, cotton, and soybean fields were also sampled from other regions along the transect where peanuts are not commonly cultivated. Regional differences in aflatoxin and cyclopiazonic acid (CPA) production by *A. flavus* were also examined.

There was considerable variability in soil populations of combined species from section *Flavi* among fields along the transect, with the highest populations occurring from central Texas to central Georgia. *A. flavus* was the dominant species within the section, and most isolates comprised the L strain. The S strain was present primarily in the cotton-growing regions of Louisiana and east-central Texas. Peanut fields had significantly lower populations of *A. flavus* compared to fields with other crops. *A. parasiticus* was most prevalent from central Alabama to Virginia, and the peanut-growing region of Georgia/Alabama had the highest populations. Soil populations of *A. parasiticus* were significantly higher in peanut fields; however, *A. parasiticus* was also present in Virginia north of where peanuts are grown. *A. tamarii* and *A. caelatus* were widely distributed but generally present at low population levels; neither species appeared to be associated with peanut cultivation. *A. nomius* was detected in only five fields from Louisiana and Mississippi.

The L and S strains of *A. flavus* differed in aflatoxin production, with the S strain producing higher concentrations and the L strain showing more variability. CPA production by L and S strains were similar, but the L strain was slightly more variable. Of the 774 L strains, 70.8% produced both aflatoxin and CPA, 16.1% produced neither toxin, and 12.4% produced CPA only. Nearly all of the S strains (n = 309) produced both toxins. The transect was divided into 18 segments of 150 to 200 km each, and differences in aflatoxin and CPA production by the L strain among transect segments were examined. Isolates from the western half of Texas and from the peanut-growing region extending from central Alabama to central Georgia produced the most aflatoxin and CPA.

POSTER PRESENTATIONS

SPREAD OF A GENETICALLY ALTERED *ASPERGILLUS* ISOLATE IN MAIZE EARS IN THE FIELD

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The spread of a transformed isolate of *Aspergillus flavus* in ears of resistant and susceptible maize hybrids was monitored in the field. An *A. flavus* strain (GAP 2-4) containing the *Escherichia coli B*-D-glucuronidase reporter gene linked to a *B*-tubulin gene promoter was inoculated into kernels using a modified pinbar technique. Ears were self pollinated and inoculations were made 21 days following pollination. Ears were harvested at 7 days after inoculation and at 7 day intervals following the first harvest. After drying ears for 7 days at 38 C, 40 kernels surrounding the wound site were removed and sliced longitudinally into halves to expose the embryo-endosperm interface. Fungal presence in the kernel halves was determined by visually assessing GUS activity using a histochemical assay. Kernels were placed cut surface down in a solution of X-gluc for 24 hrs and held at 38 C. Internal kernel tissues (embryo and endosperm) were examined for blue color and rated on area with GUS activity. Fungal spread was slower in resistant hybrids compared to a susceptible hybrid. At 14 days after inoculation, *A. flavus* (GUS activity) was detected in 65 and 67% of the kernels surrounding the inoculation site from the resistant hybrids; however, all kernels from the susceptible hybrid were infected with *A. flavus*. GUS activity in kernels from both resistant and susceptible hybrids was found almost exclusively in the embryo region. GUS activity in the embryo was concentrated in the scutellum with very little activity in the coleoptile region. Cob sections from the susceptible hybrid stained with X-gluc had GUS activity at 7 and 14 days after inoculation. GUS activity in the cob sections was limited to vascular tissue in the rachis and rachilla regions. No GUS activity was observed in cob sections from the susceptible hybrid harvested after 14 days and no GUS activity was found in any cob sections from resistant hybrids.

GENOTYPIC DIVERSITY OF *ASPERGILLUS PARASITICUS* IN AN ILLINOIS CORN FIELD

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Aspergillus parasiticus was isolated from direct platings of soil from a corn field near Kilbourne, Illinois. Soil contained 0.2-4.0 CFU/g of *Aspergillus flavus* and/or *A. parasiticus*. Sixty isolates, each one from a separately collected soil sample, were examined for ability to produce sclerotia and aflatoxins, and were submitted to DNA fingerprinting. Pst I digests of total genomic DNA from each isolate were probed using the pAF28 repetitive sequence. Among 60 isolates analyzed, 42 distinct DNA fingerprint groups were identified (each group sharing less than 80% pAF28 band similarity). *A. parasiticus* genotype number 8 represented 12% of the sample population. The 70% genotypic diversity of the *A. parasiticus* population was only marginally lower than the 84% genotypic diversity recorded earlier for a population of *A. flavus* isolates from the same field soil. Sclerotia were produced by 83% of the *A. parasiticus* genotypes during dark incubation (25°C). All isolates of *A. parasiticus* produced aflatoxin B_1B_2 and G_1G_2, whereas only 42% of the *A. flavus* genotypes isolated from these soils produced aflatoxins.

INTERACTIONS OF SAPROPHYTIC YEASTS WITH *ASPERGILLUS FLAVUS* IN WOUNDED PISTACHIOS

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Aflatoxins are toxic and carcinogenic compounds produced by the fungus *Aspergillus flavus* and *Aspergillus parasiticus*. Contamination by aflatoxin in tree nuts, peanuts, corn and cottonseed has been recognized as a serious food safety hazard to both human beings and animals. *A. flavus* is a wound invading pathogen infecting pistachios and almonds which have been damaged by insects, animals, early splits and mechanical harvest. Any established infection of *A. flavus* or *A. parasiticus* will result in rapid accumulation of aflatoxin in the harvested nuts under warm temperature and highly humid conditions.

Removing aflatoxin from contaminated edible nuts is impractical and expensive. Research is, therefore, needed to develop intervention strategies designed to control infection of tree nuts by *A. flavus*. Saprophytic yeasts which can colonize plant surfaces for a very long period of time under dry conditions produce extracellular polysaccharides that enhance their survivability and restrict both colonization sites and nutrient flow to other fungi. The potential of yeasts as effective biocontrol agents against *A. flavus* is being investigated in current projects.

Aflatoxins are synthesized via the polyketide metabolic pathway involving more than twenty five genes. The first stable intermediate is norsolorinic acid. At least 16 enzyme-catalyzed steps are required to complete the synthesis of aflatoxin B1 from norsolorinic acid. The NOR mutant of *A. flavus* has a block in the reductase and is unable to complete aflatoxin biosynthesis, instead the mutant accumulates norsolorinic acid, a bright red orange, easily visualized compound. *A. flavus* 827, a white spored NOR mutant, has been used in this study as an indicator strain for monitoring the interactions of yeast with *A. flavus*.

When selected yeast strains were applied to pistachios wounded and inoclulated with the NOR mutant, the growth and sporulation of the fungus were reduced considerably. The number of conidia produced on the pistachios was reduced by 50 to 70%. Accumulation of norsolorinic acid was inhibited because no visible red orange color was observed. The result suggests that the aflatoxin production by *A. flavus* was prevented by the yeasts.

EXTRACTION, DETECTION AND ANALYSIS OF AFLATOXINS

POSTER PRESENTATIONS

THE USE OF SUPERCRITICAL FLUIDS FOR THE EXTRACTION AND ANALYSIS OF AFLATOXINS

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Supercritical fluids are finding increased as replacements for organic solvents in the extraction of mycotoxins from a variety of sample matrices. In this presentation, we report on our experiences in utilizing supercritical fluid carbon dioxide ($SC\text{-}CO_2$) with an appropriate cosolvent for the remov-al of aflatoxin B_1 from yellow/white corn, and aflatoxin M_1 from bovine liver.

Supercritical fluid extraction (SFE) was performed with an Isco SFX 2-10 extractor, or alteratively on a larger scale extractor fabricated at NCAUR. Extractions were conducted on neat, sieved corn fractions from 3-50 grams, while beef liver extractions were performed by mixing the liver puree with a sample dispersant, Hydromatrix. Pressure and temperatures up to 15,000 psi and 150°C were utilized in order to optimize the recovery via SFE.

Neither neat SC-CO2 nor high pressure were sufficient to achieve high recoveries of B_1 from yellow dent corn. Optimal extraction of B_1 was accomplished at 5000 psi and 80°C using a 2:1/acetonitrile:methanol cosolvent at 15 volume %. Levels of aflatoxin B_1 in the yellow dent corn were found to depend on the sample size and age of the sample, whether SFE or con- ventional solvent extraction were employed. Extrapolation of the above extraction conditions to white corn samples did not result in equivalent recoveries of B_1 at equal levels of con- tamination and sample size.

Supercritical fluid or organic solvent extraction of M_1 from bovine liver was complicated by high levels of coextractives. However careful adjustment of the SFE conditions, particularly the amount of the fluid/cosolvent utilized, resulted in M_1 recoveries equivalent to those achieved with organic solvent. Both extracts however,required cleanup prior to liquid chromatographic analysis.

A RAPID FLUOROMETRIC TEST FOR THE QUANTITATION OF AFLATOXIN IN CORN, NUTS AND COTTONSEED

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Aflatoxin testing of ingredients has become an integral part of U.S. feed and food companies acceptance criteria. To routinely accept or reject ingredients prior to unloading requires a rapid analysis.

A rapid, quantitative, inexpensive, and efficient method to determine aflatoxins in corn, peanuts, cottonseed and other grains was developed.

This method has been successfully applied to corn, peanuts, cottonseed and other grain matrixes. The sensitivity of the method is 5 ppb of total aflatoxin and the linear range is 5000 ppb with no dilution.

The method was validated by analyzing non-contaminated samples spiked with known amounts of aflatoxin and by the comparison of naturally contaminated samples with HPLC analysis. Good correlation was obtained with the fluorometric and HPLC results for aflatoxin in corn, peanuts and cottonseed with r^2 of 0.999, 0.980, and 0.999, respectively.

REGULATION OF AFLATOXIN BIOSYNTHESIS

POSTER PRESENTATIONS

MOLECULAR CHARACTERIZATION OF THE EFFECTS OF NUTRITIONAL FACTORS ON TRANSCRIPTION AND AFLATOXIN PRODUCTION BY REPORTER STRAINS OF ASPERGILLUS PARASITICUS

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The elimination of aflatoxin from the food chain is desirable due to economic and health concerns. To that end, the molecular approaches have identified and characterized several genes involved in aflatoxin biosynthesis. To investigate the mechanisms governing the expression of aflatoxin genes, we have grown *Aspergillus parasiticus* reporter strains in a series of media designed to induce or repress aflatoxin production. Glucose minimal salts (GMS) supports aflatoxin production by D8D3 (*nor*-1/*uid*A) and Isolate 4 (*ver*-1/*uid*A) provided zinc is added to the medium. Nitrate minimal salts (NMS) and Peptone minimal salts media do not support aflatoxin production. Changes in GUS activity mimics changes in aflatoxin production, further supporting the belief that aflatoxin biosynthesis is regulated at the level of transcription. The relationship between aflatoxin production and GUS activity also establishes these fusion strains as reliable indicators of expression in these media. Furthermore, variations in aflatoxin production were also mirrored by changes in the level/presence of Nor-1 and Ver-1. The levels of Afl-R, however, did not change in the same manor, rather they remained constant for each medium. These data suggest that though aflatoxin production does not occur without Afl-R, Afl-R alone is not sufficient to induce aflatoxin production.

THE IMMUNODETECTION OF GENE PRODUCTS INVOLVED IN AFLATOXIN BIOSYNTHESIS

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Aflatoxins are highly toxic and carcinogenic secondary metabolites produced by certain species of filamentous fungi including *Aspergillus parasiticus* and *Aspergillus flavus*. Many of the genes involved in aflatoxin biosynthesis have been identified and are clustered in 60-kb genomic DNA fragment. AflR, regulatory factor of this gene cluster, and structural genes including *pks*A, *nor*-1, *vbs*, *ver*-1, *omt*A and *ord*-1 are necessary for aflatoxin biosynthesis. Our working hypothesis is that aflatoxins are concentrated at specific fungal structures, for example, sclerotia and spores which are important for fungal survival and colonization. In this present work, we demonstrated a 10 fold higher AFB1 concentration in spores than in a whole colony or sclerotia by using direct competitive ELISA (based on one experiment). Indirect immunofluorescence microscopy demonstrated that OmtA protein localized in conidial heads (or premature spores) and in the mycelium at 48 h grown on YES agar. VBS also localized at or near the conidial head. AflR and Ver-1 seemed to localize in the conidiophore. Western blot analysis showed that AflR and OmtA proteins accumulate by 24 h in liquid culture. Other structural genes products, including VBS, Nor-1 and Ver-1, accumulate by 48 h and are maintained at this same level through 72 h. In conidia (harvested at 8 days), the concentration of Nor-1 and Ver-1 are much less than AflR and OmtA. In sclerotia, isolated after 7 days growth on PDA, AflR and Nor-1 showed lower concentration than the whole colony. The lower concentration of Nor-1 and Ver-1 proteins in spores and sclerotia may be due to down regulation of these early pathway gene products in late stage of development.

CHARACTERIZATION OF AFLR, A REGULATORY PROTEIN INVOLVED IN AFLATOXIN BIOSYNTHESIS, IN *ASPERGILLI*

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The role of the regulatory gene *aflR* and its gene product, AFLR, in the biosynthesis of aflatoxin in various *Aspergillus* species was studied. Western blot, ribonuclease protection assay (RPA) and ELISA analyses of selected aflatoxigenic *Aspergilli* (i.e., *A. parasiticus* and *A. flavus*) and non-aflatoxigenic *Aspergilli* (i.e. *A. flavus*, *A. sojae* and *A.oryzae*) species revealed that both *aflR* mRNA and AFLR protein were present in all the examined species; however, the *omtA* transcript, encoding a 42 kDa sterigmatocystin-*O*-methyltransferase, involved in the latter stages of the aflatoxin biosynthetic pathway, was not detected in any of them. AFLR in *A. oryzae* was found to be regulated by carbon source and temperature similar to the regulatory profile of AFLR in *A. parasiticus.* To Identify the factor(s) involved in the inhibition of aflatoxin formation in non-aflatoxin producer, protein extracts obtained from aflatoxigenic *A. parasiticus* NRRL 2999, non-aflatoxigenic *A. caelatus* 93SZ5 and *A. oryzae* NRRL 451 mycelial mass were partially purified through ultracentrifugation, hydroxyapatite (HA) chromatography and DEAE-cellulose chromatography. Western blotting using AFLR specific antibodies as the probe showed that AFLR was eluted from the DEAE-cellulose column with 0.15 M NaCl. Electrophoretic mobility shift assay (EMSA) was then used to study the DNA (oligonucleotide containing sequence TTAGGCCTAA) binding ability of these partially purified protein extracts. The results of EMSA showed that the protein-DNA binding complex was only found in HA column- purified protein extracts obtained from *A. parasiticus*, but not in protein extracts obtained from *A. caelatus* and *A. oryzae.* The protein present in the protein-DNA complex was identified to be AFLR by employing antibodies specific to AFLR in an antibody supershift assay. Our findings suggest that the presence of AFLR in non-aflatoxigenic *Aspergilli* can not turn on the structural gene, *omtA*, and aflatoxin production; it is probably caused by the failure of AFLR protein to bind to the specific DNA fragment.

IS AN AFLR-REPRESSOR INTERACTION INVOLVED IN NITRATE INHIBITION OF AFLATOXIN BIOSYNTHESIS IN ASPERGILLUS PARASITICUS?

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A variety of nutrients and environmental factors affect aflatoxin production in *Aspergillus parasiticus*. Nitrate added as the sole nitrogen source completely inhibits aflatoxin production. In *A. parasiticus* strains transformed with the aflatoxin pathway regulatory gene, *aflR*, nitrate inhibition is alleviated.

To test if the AFLR carboxy terminus plays a role in the derepression, we fused a DNA fragment encoding this region to the *A. parasiticus* nitrite reductase gene (*niiA*) promoter and transformed this construct, *niiA::aflRC*, into *A. parasiticus* RHN1, an OMST-accumulating strain. Two types of transformants were obtained from Czapek Solution Agar (CZ) plates; one was lightly pigmented and another nonpigmented. Southern hybridization and PCR analyses showed that the pigmented transformants contained two copies of *niiA:aflRC*; one was integrated into the *niaD* locus and another into the *aflR* locus of the fungal genome. In contrast, the nonpigmented transformants contained one copy of *niiA:aflRC* which was integrated into the *niaD* locus.

Semi-quantitative thin layer chromatography analysis showed that the level of the precursors and OMST in the pigmented transformants grown on CZ plates was comparable to that in the parental strain, SRRC 2043, grown on Potato Dextrose Agar plates. On the contrary, no precursors and OMST were produced in SRRC 2043 grown on CZ plates. These results suggest that a threshold of *aflR* gene product is needed for the alleviation of nitrate inhibition. A model based on the interaction of the AFLR protein and a repressor protein is proposed.

SITE-DIRECTED MUTAGENESIS OF THE AFLATOXIN BIOSYNTHESIS REGULATORY GENE, *aflR*

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The gene, *aflR*, encodes a Cys_6-zinc cluster, sequence-specific, DNA-binding protein that is required for regulation of the aflatoxin biosynthetic pathway in *Aspergillus spp* (1,5). Previously we found that transformation of *A. parasiticus* with a plasmid containing an intact copy of *aflR* resulted in overproduction of aflatoxin pathway metabolites (2). In the present study, site-directed mutagenesis was used to alter several regions in the coding portion of the gene homologous to sequences encoding protein segments necessary for the activity of other GAL4-type transcription factors (3,4). *A. parasiticus* transformants containing copies of *aflR* with a T→G transversion designed so that the final Cys in the Cys_6 domain is replaced by a Trp had a 100-fold lower metabolite biosynthesis than did the untransformed strain. Deletion of the putative nuclear localization signal region by a 15-bp deletion caused a similar reduction in metabolite biosynthesis. Non-conservative changes of two basic amino acids in the putative DNA-binding specificity region carboxy terminal to the Cys_6 cluster also gave transformants with reduced metabolite production. In all of these transformants reduction of metabolite production was proportional to mutated gene copy number, suggesting that cross-dimerization of mutant AFLR with wild-type AFLR gives protein unable to activate aflatoxin pathway biosynthesis. These results demonstrate that AFLR is essential for aflatoxin biosynthetic pathway regulation and behaves similarly to other pathway Cys_6 regulatory proteins from fungi and yeast.

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MOLECULAR AND GENETIC ANALYSIS OF AFLATOXIN REGULATORY MUTANTS IN *ASPERGILLUS FLAVUS*

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The molecular component of the research conducted in our lab is comprised of three major objectives: (1) Characterization of the Zn(II) 2Cys6 binuclear cluster DNA binding regulatory gene *aflR*, and its binding to and transcriptional activation of aflatoxin biosynthetic pathway genes in *Aspergillus flavus*; (2) Determination of the function of the *aflJ* gene product required for aflatoxin biosynthesis, and the regulation of its expression; (3) Elucidation of the upstream regulatory hierarchy governing the transcriptional control of aflatoxin biosynthesis in *Aspergillus flavus*. To date, we have identified putative transactivation domains, poly(A) adenylation signals, and dimerization motifs for *aflR*, and are in the process of characterizing these sites by site-directed mutagenesis. The binding of the *nor-1* structural gene promoter by *aflR* has been investigated by electrophoretic mobility shift assays and deletion analysis of the *nor-1* promoter fused to the GUS reporter gene. We have identified a putative binding site for *aflR* in the region spanning -103 to -88 of the 5'-UTR of the *nor-1* promoter. We have also investigated the transcriptional expression of the *aflJ* gene, which is required for aflatoxin biosynthesis but contains no identifiable motifs for enzymatic or DNA-binding activity. The expression of *aflJ* appears to be regulated similarly to aflatoxin pathway genes, and we are characterizing the promoter region of this gene for regulatory elements. We also have begun to characterize the upstream regulation of aflatoxin biosynthesis. We have identified three putative regulatory mutant strains which produce no identifiable *aflR* transcript and fail to express an *aflR*(pro)::GUS construct. Expression of *aflR* from the constitutive *A. nidulans gpdA* promoter restores aflatoxin biosynthesis in these strains. We are in the process of identifying the genes mutated in these strains by complementation. The long term goal of this research is to understand the regulation of aflatoxin biosynthesis in *A. flavus* and to identify potential targets for the control of aflatoxin contamination of important commodities. The identification of corn kernel constituents exhibiting inhibitory activity to both *A. flavus* growth and aflatoxin biosynthesis highlights the importance of identifying target sights for this control activity. Additionally, in the course of this research, reporter and expression constructs have been generated which have proven valuable as tools in monitoring fungal growth and aflatoxin biosynthesis under field conditions.

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INSIGHTS INTO REGULATION OF AFLATOXIN BIOSYNTHESIS FROM AN UNUSUAL *ASPERGILLUS FLAVUS* STRAIN FROM AFRICA

P. J. Cotty[1], T. Feibelman[1], and K. F. Cardwell[2], [1]USDA, ARS, Southern Regional Research Center, USDA, ARS, New Orleans, LA 70179, and [2]Biological Control Center for Africa, IITA, Cotonou, Republic of Benin

An unusual aflatoxin producing fungus was isolated from soil collected in the Republic of Benin. This fungus differed from other aflatoxin producing fungi in both sclerotial morphology and regulation of aflatoxin biosynthesis. The fungus (called Strain P) was assigned to *A. flavus* based on conidial morphology and polymorphisms in the Taka-amylase gene. Strain P produced aflatoxins B_1 and B_2 and elongate sclerotia up to 2 cm in length with a bulbous base and reduced melanin content. Only one fungal isolate with a morphology similar to Strain P was observed among over 900 isolates from west Africa and none has been observed among several thousand that have been examined in North America. Thus strain P probably is not and important etiologic agent of aflatoxin contamination of crops. However, the regulation of aflatoxin biosynthesis in strain P may shed light on aspects of the regulation of aflatoxin production in all aflatoxin producing fungi. Unlike other *A. flavus* strains, aflatoxin biosynthesis by Strain P was inhibited by ammonium and stimulated by nitrate. Aflatoxin production by Strain P was directly correlated with final culture pH in ammonium medium. This response is reverse that of other *A. flavus* strains. Thus, nitrogen source influences may reflect an altered response to pH. Although previous work suggested that sclerotial morphogenesis and aflatoxin biosynthesis may have interrelated regulation, it is unknown if the distinct sclerotial morphology of strain P and its altered regulation of aflatoxin biosynthesis result from the same regulatory change. Strain P may be useful in the study of aflatoxin regulation.

USE OF SITE-DIRECTED MUTAGENESIS FOR DERTERMINATION OF AMINO ACID RESIDUES CRITICAL FOR OXIDOREDUCTASE ACTIVITY INVOLVED IN AFLATOXIN BIOSYNTHESIS

J. Yu[1], K. C. Ehrlich[1], P.-K. Chang[2], B. Montalbano[1], D. Bhatnagar[1],and T. E. Cleveland[1], [1]USDA, ARS, Southern Regional Research Center, New Orleans, LA; and [2]Tulane University, New Orleans, LA

Aflatoxins (B_1, G_1 B_2 and G_2) are toxic and carcinogenic secondary metabolites. The aflatoxin biosynthetic pathway involves at least 18 enzymatic reactions(1-11). In the later stage of aflatoxin biosynthesis, the conversions of *O*-methylsterigmatocystin (OMST) to aflatoxin B_1 and G_1, and dihydro-*O*-methylsterigmatocystin (DHOMST) to aflatoxin B_2 and G_2 have been shown to be carried out by an oxidoreductase in *Aspergillus parasiticus* and *A. flavus* (1, 2).

We have cloned the *A. parasiticus ordA* gene, capable of encoding a protein of 528 amino acids with a calculated molecular mass of 60 kDa; the gene consists of seven exons and six introns and is identical to the *A. flavus ordI* gene cloned earlier. The deduced protein sequence contains a highly conserved motif present in all cytochrome P-450 type enzymes with the conserved amino acid residues of "**F - - G - - - C - G** " and of "**E - - R** ". The highly hydrophobic nature of over 20 amino-terminal residues, which is postulated to serve as a membrane spanning anchor, was consistent with all other microsomal P-450 enzymes.

Southern and Northern blot analyses of the OMST-accumulating mutant strain (*A. parasiticus* SRRC 2043) showed that there was no apparent deletion in the *ordA* gene and that the *ordA* gene was transcribed in this mutant strain as well. Complementation by transformation of the wild type *ordA* gene construct into the *O*-methylsterigmatocystin-accumulating mutant strain (SRRC 2043) restored the strain's ability to convert OMST and DHOMST to aflatoxin B_1 and G_1, and B_2 and G_2, respectively. Additionally, galactose-induced expression of the *ordA* gene in the *Saccharomyces cerevisiae* under the transcriptional control of the *gal1* promoter demonstrated that the *ordA* gene was able to convert exogenously supplied OMST to aflatoxin B_1; while the *ordA* gene cloned from the mutant strain SRRC 2043, was unable to carry out the same conversion.

The sequence data revealed that the two sequences of the *ordA* gene from thewild type and mutant strains were almost identical except for a few base changes resulting in the alteration in three amino acids in the oxidoreductase from the mutant strain. It was determined that these point mutations disabled the enzymatic activity of the oxidoreductase. Studies by site-directed mutagenesis demonstrated that His-400 is the most critical amino acid for the enzymatic activity. When His-400 was mutated to Leucine (as in the mutant strain), no conversion from OMST to aflatoxin was detected. Ala-143, also plays a significant role in enzyme activity but not as critical as His-400. When Ala-143 was mutated to a serine, the conversion capability was significantly reduced by approximately 50%. Ile-528 may also be required for enzyme activity because when leucine 400 (in the mutant strain) was mutated

back to histidine (wild type), the enzymatic activity was not restored. We, therefore, conclude that: a), The *ordA* gene encodes a fungal cytochrome P-450 type oxidoreductase, which can convert OMST to AFB1 as demonstrated in the yeast expression system; b), the *ordA* gene product is involved in the conversions of OMST and DHOMST to aflatoxins (B_1, G_1, B_2, and G_2) in the aflatoxin biosynthetic pathway in *A. parasiticus*; and c), the His-400, Ala-143, and Ile-528 are significant components of the active site of this enzyme.

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SPECIAL PRESENTATION

Sara H. Henry, Food and Drug Administration, Washington, D.C.

Summary of Presentation:

AFLATOXIN AND THE INTERNATIONAL SCENE

Sara Hale Henry, Ph.D.
Contaminants Branch
Center for Food Safety & Applied Nutrition
U.S. Food & Drug Administration

FDA Regulatory Total Aflatoxin Levels

Commodity	Concentration (ug/kg)
All products for humans (except milk)	20
Corn – immature animmals & dairy cattle	20
Corn/peanuts for swine, breeding cattle, mature poultry	100

c

FDA Regulatory Total Aflatoxin Levels

Commodity	Concentration (ug/kg)
Corn/peanuts – finishing swine	200
Corn/peanuts – finishing beef cattle	300
Cottonseed meal (feed)	300
All other feedstuffs	20
Milk	0.5 (as AFM1)

Medians & Ranges of Maximum Tolerated Levels (ug/kg) of Aflatoxins (1994)

Commodity	Median (ug/kg)	Range (ug/kg)
B1 in foods	4	0-30
B1 in feeds	20	5-1000
M1 in milk	0.05	0-1

Proposed European Commission Regulations (Aug. '97)

- Groundnuts, nuts & dried fruits 2 ug/kg B1
  4 ug/kg B1, B2, G1, G2
- Same - unsorted 5 ug/kg B1
  10 ug/kg B1, B2, G1, G2
- Cereals & processed cereals 2 ug/kg B1
  4 ug/kg B1.,B2, G1, G2
- Milk & milk products 0.05 ug/kg M1

Codex Alimentarius

- **Funded by World Health Organization & Food & Agriculture Organization**
- **Involves 150 countries**
- **Purposes are to facilitate world trade & protect public health**
- **Lead US rep. is from USDA**

World Trade Organization

- Dispute resolution body for Codex
- Stronger after Uraguay round of GATT
- Sanitary & Phytosanitary Agreement
  - rule-based
  - not based on social policy/environmental issues

US vs. European Approach to Codex

- US prefers rule-based
- Europeans want consideration of social policy & environmental issues
  - e.g., milk pasteurization dispute between US & Europe, primarily France

Joint FAO/WHO Expert Committee on Food Additives

- Provides scientific advice to Codex
- Made up of international scientific experts
- Made up of permanent members & temporary advisers
- Charged to present science-based only advice

JECFA Purposes

- Elaborate principles for evaluating safety of food additives
- Undertake toxicological evaluations of certain food additives & contaminants

JECFA Purposes (cont'd)

- Review & prepare specifications for selected food additives
- Assess the intake of selected food additives and contaminants

JECFA and Aflatoxins

- Considered aflatoxin to be a potential human carcinogen, in keeping with IARC
- Urged that intake of dietary aflatoxins be reduced to lowest practicable levels.

New JECFA Approach to Aflatoxins

- **Sought by Codex**
- **Involved interdisciplinary sub-committee**
  - **S.H. Henry, US FDA, Chair**
  - **F.X. Bosch, Cancer Epid. Unit, Hosp. Duran I Reynals, Barcelona, Spain**
  - **C.J. Portier, US NIEHS**
  - **assisted by J. Wilson, U.S. Resources for the Future**

Table 7. Causal factors of liver cancer and estimates of the attributable fractions.

Factor	low risk countries in Europe, and the US		Japan		high risk countries in Africa and Asia	
	Estimate	Range	Estimate	Range	Estimate	Range
Hepatitis B	<15%	4-50%	20%	18-44%	60%	40-90%
Hepatitis C[3]	60%	12-64%	50%	40-80%	< 10%	NE
Aflatoxin	limited exposure		limited exposure		important exposure[1]	
Alcohol	< 15%[4]		< 20%	11-30%[5]	NE	
Tobacco	< 12%[4]		40%	38-51%[5]	NE	
Oral contraceptives		10-50%[2]	NE		NE	
Other	< 5%				< 5%	

(1): attributtable risk not quantified
(2): restricted to liver cancer in women. Likely to increase in future generations. Uncertain if hepatits infections (notably HCV) are a necessary co-factors.
(3): not including double infections with HBV and HCV. Very few studies available using second generation assays.
(4): estimates for the US.
(5): estimates from three estudies of LC in men.
NE: non evaluated.
Note: attributable fractions do not necessarily add to 100% due to multiple exposures and possible interactions between risk factors.

Adapted from refs 46,63,78,79.

Attachment to part I of working paper on aflatoxins (PCS/FA/97.17)

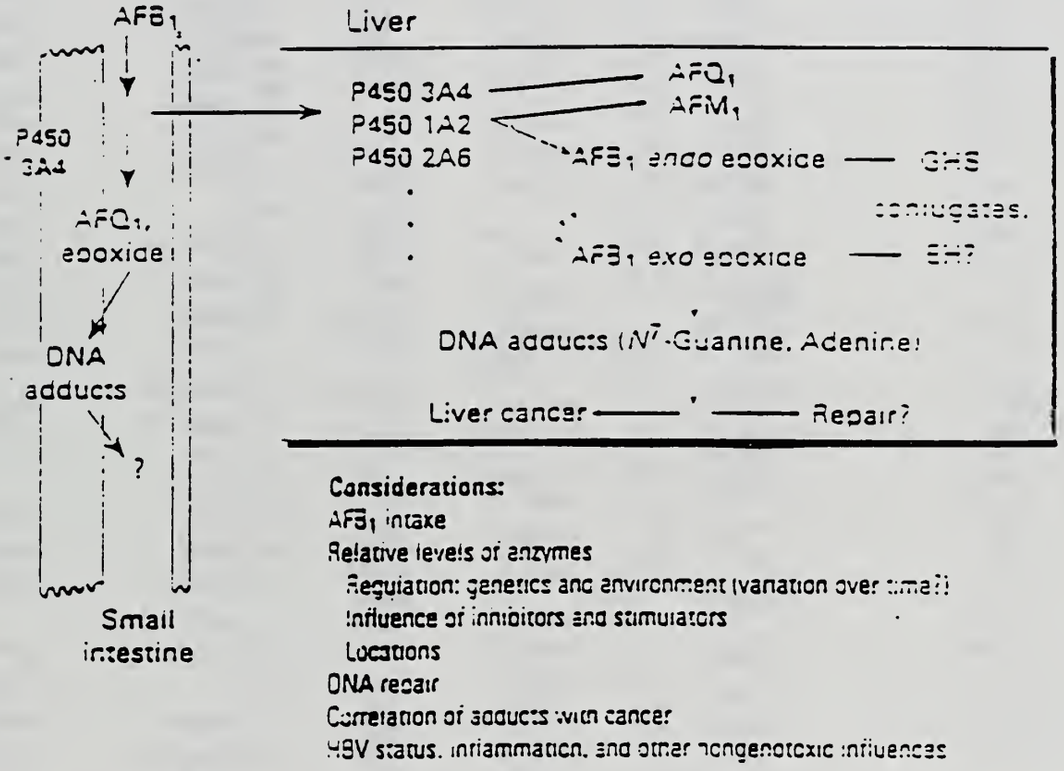


Figure 1. Complications involved in the metabolism of AFB_1 and relevance to hepatocellular cancer.

Viral Infection/Liver injury

Some data indicate that viral infection and liver injury alter expression of carcinogen-metabolizing enzymes.

Important Factors in Species/strain Sensitivity to Aflatoxins

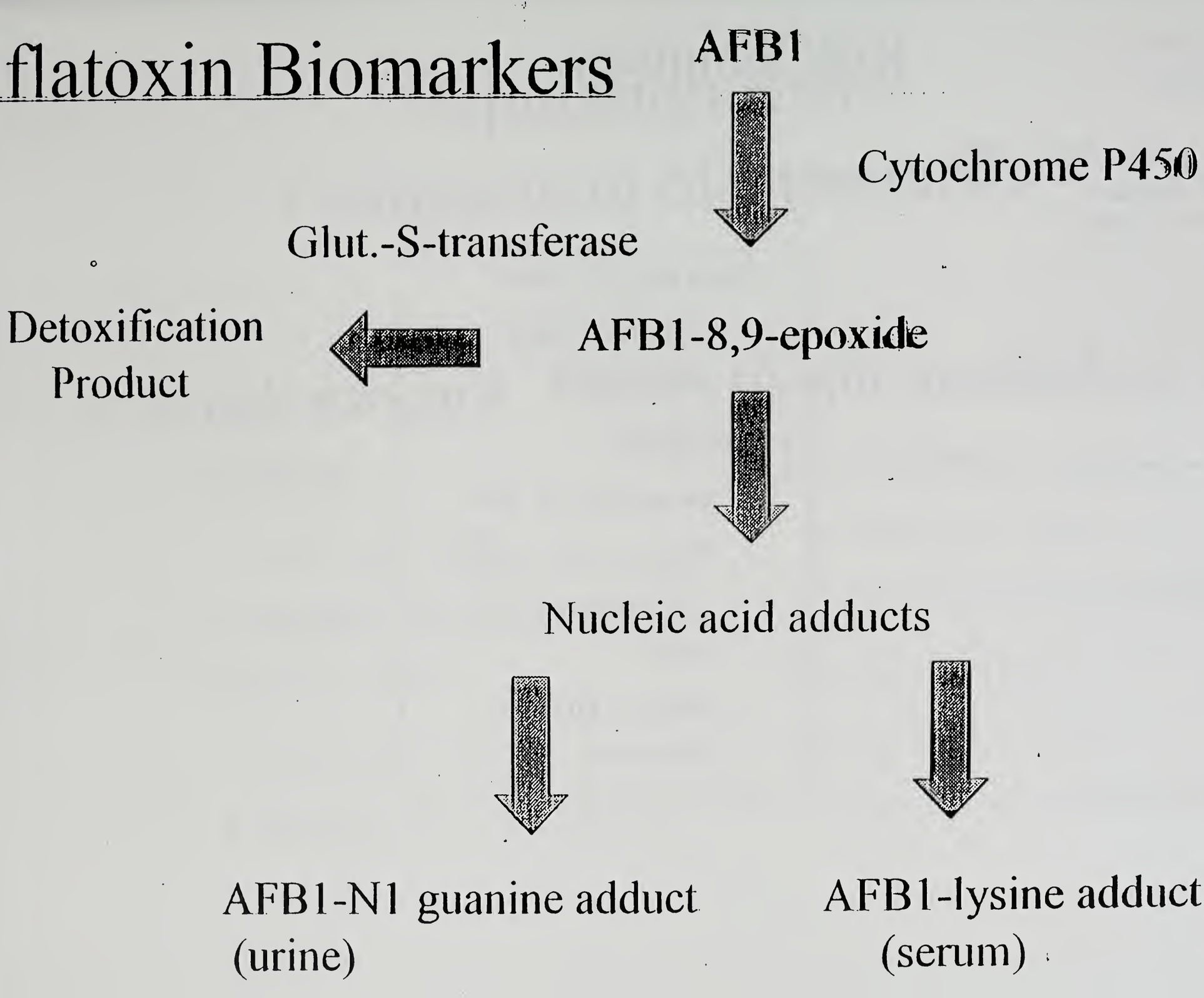
- **Proportion of AFB1 that is metabolized to 8,9-epoxide relative to other less toxic metabolites**
  - **Genetic variation in Phase I enzymes**
- **Relative activity of Phase II enzymes (GSTs) which form non-toxic conjugates and inhibit cytotoxicity**
- **Some data exist indicating a correlation between AFB1-DNA adduct concentrations and GST mu class polymorphisms**

Toxicity of AFM1 vs. AFB1

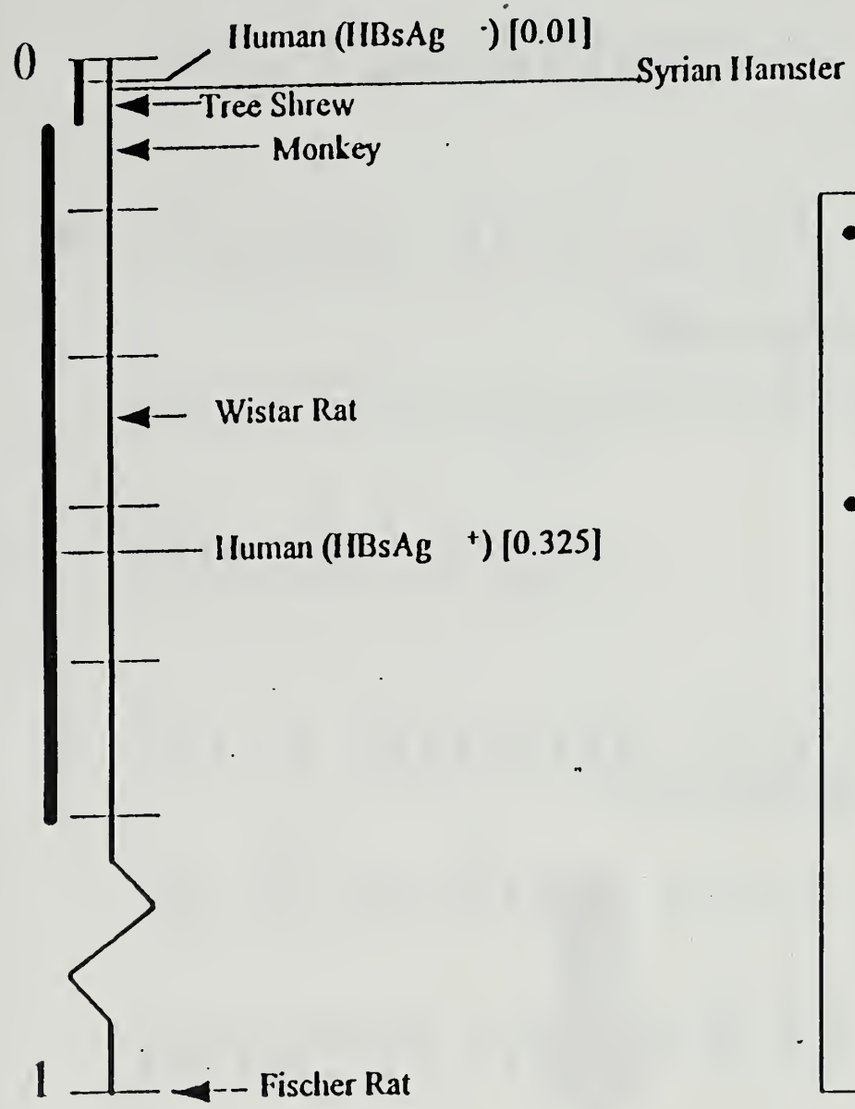
- Acute toxicity of AFM1 & AFB1 nearly equivalent in rats & ducklings
- In a tumor-incidence comparison in Fischer rats, AFM1 is 2 - 10% as potent as AFB1.

(*from Cullen et al., 1987*)

Aflatoxin Biomarkers



Risk Summary



- Risk may be zero
  - negative studies
  - confounding
- Concerns
  - inaccurate AF dose
  - Error in HBV status
  - small numbers in + study
  - shape of HBV/AF interaction

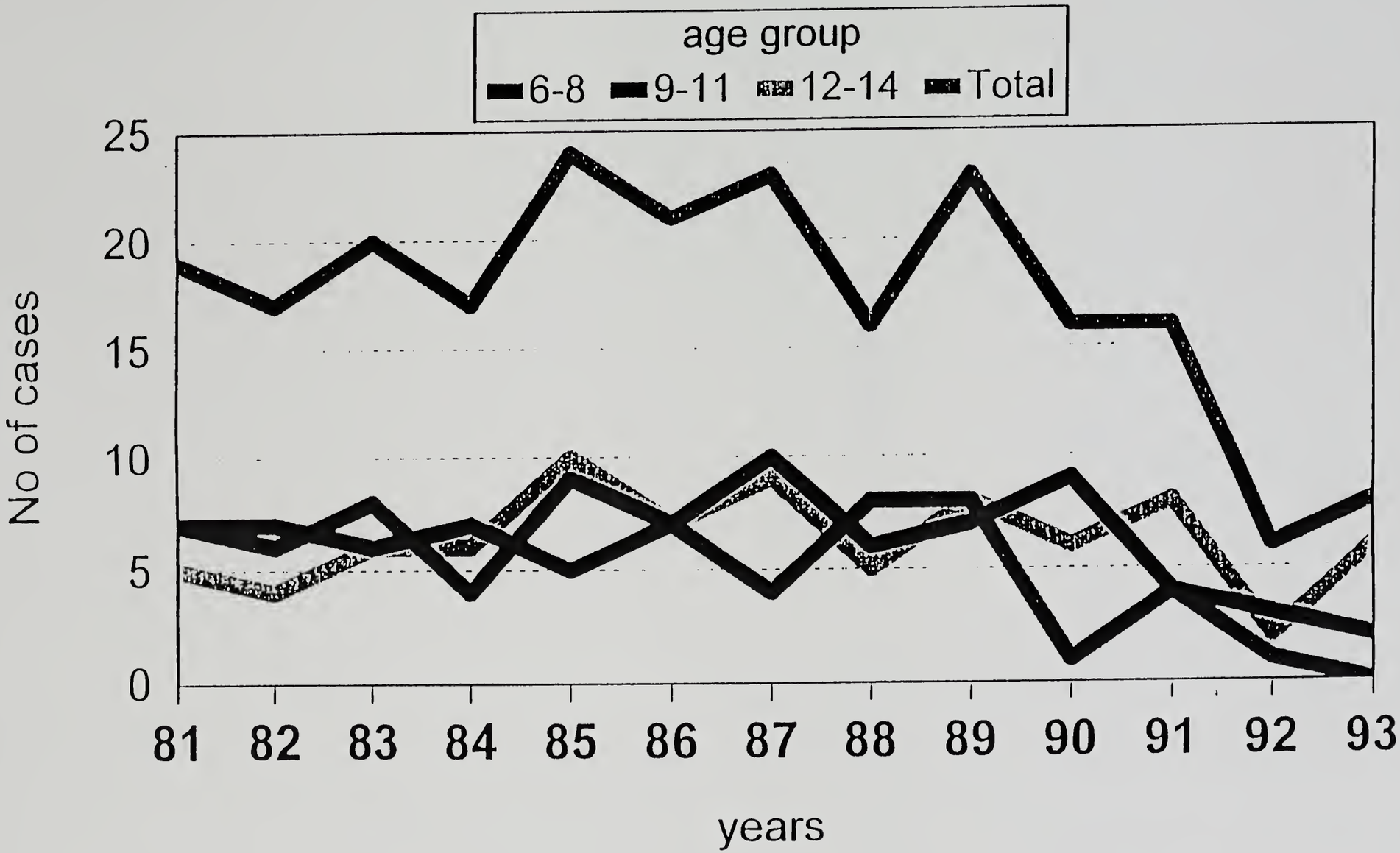
Population Risks
Comparison of Standards

- **20 ppb standard**
  - US Risk
    - 0.003 per 100k
    - discard 7% of samples
  - Gambia Risk
    - 0.382 per 100k
    - discard 25% of samples
- **10 ppb standard**
  - US Risk
    - 0.002 per 100k
    - discard 10% of samples
  - Gambia Risk
    - 0.234 per 100k
    - discard 40% of samples

CONCLUSIONS

- Potency estimates have been derived from limited human data
  - use extreme caution until verification
  - ongoing studies should address this shortly
- Lowering current western standards on aflatoxin will not result in a detectable change in the incidence of liver cancer
- Lowering aflatoxin standards in high risk countries would probably result in a reduction in liver cancer among carriers of HBV
  - HBV vaccines may have a better chance of lowering overall liver cancer rates

Number of liver cancer cases registered in Taiwan.



APPENDIX I

AGENDA
1997 AFLATOXIN ELIMINATION WORKSHOP
OCTOBER 26-28, 1997
MEMPHIS, TN

SUNDAY, OCTOBER 26, 1997

5:00-8:00 p.m. REGISTRATION / POSTER ASSIGNMENTS

6:00 p.m. POSTER VIEWING
(Posters will be available for viewing for duration of the meeting beginning Sunday, October 26th.)

6:00-8:00 p.m. MIXER

MONDAY, OCTOBER 27, 1997

7:00 a.m. REGISTRATION / POSTER ASSIGNMENTS

8:00 a.m. WELCOME: Phillip Wakelyn, National Cotton Council

INTRODUCTORY REMARKS: Jane F. Robens, USDA/ARS/National Program Staff

SESSION 1: **CROP MANAGEMENT AND HANDLING, INSECT CONTROL AND FUNGAL RELATIONSHIPS**
Chair: Dave Ramos, Walnut Marketing Board

8:15 a.m. **"Timing of aflatoxin contamination of cottonseed in south Texas in relation to boll maturity."** Tom Isakeit, Texas A&M University, Weslaco, TX.

8:30 a.m. **"Aflatoxin contamination in cottonseed at weekly intervals in modules from Arizona, Mississippi and Texas."** William E. Batson, Jr.,[1] Jacobo Caceres,[1] Peter J. Cotty,[2] and Tom Isakeit.[3] [1]Mississippi State University, Mississippi State, MS; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA; and [3]Texas A&M University, Weslaco, TX.

8:45 a.m. **"Grower practices that reduce aflatoxin contamination of figs and pistachio nuts."** Themis J. Michailides, University of California, Riverbend, CA.

9:00 a.m. **"Detection, sorting and testing of aflatoxin contaminated pistachios and almonds."** Tom F. Schatzki, Tom C. Pearson and Lau-Chau Le, Ray Miller and Martin Ong, USDA, ARS, Western Regional Research Center, Albany, CA.

9:15-10:00 a.m. **PANEL DISCUSSION**
Panel Chair: Patrick F. Dowd

10:00-10:30 a.m. **BREAK**

SESSION 2: **POTENTIAL USE OF NATURAL PRODUCTS FOR PREVENTION OF FUNGAL INVASION AND/OR AFLATOXIN BIOSYNTHESIS IN CROPS**
Chair: Howard Valentine, American Peanut Council

10:30 a.m. **"The role of natural products, semiochemicals and microbial agents in reducing insect infestations, *Aspergillus* infection and aflatoxigenesis in tree nuts."** Bruce Campbell, Nelson Goodman, Sylvia Hua, Douglas M. Light, Russel J. Molyneux, J. Roitman, Noreen Mahoney, G. Merrill, James Baker, and Christopher Mehelis, USDA, ARS, Western Regional Research Center, Albany, CA.

10:45 a.m. **"Interactions between sugars, sugar metabolites and triglycerides in the production of aflatoxin by *Aspergillus flavus* NRRL 3357."** Robert A. Norton, USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

11:00 a.m. **"Soybean lipoxygenase is active on non-aqueous media at low moistures: A constraint to xerophilic fungal growth."** Harold W. Gardner, USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

11:15 a.m. **"The effects of growth conditions and development on aflatoxin synthesis."** John Linz, Michigan State University, E. Lansing, MI.

11:30-1:30p.m. **LUNCH / POSTER VIEWING**

1:30 p.m. **"Gene expression systems in aflatoxigenic fungi for monitoring aflatoxin elaboration in crops."** Deepak Bhatnagar, USDA, ARS, Southern Regional Research Center, New Orleans, LA.

1:45 p.m. **"Immunochemical studies on regulation of aflatoxin formation."** Bing H. Liu and Fun S. Chu, University of Wisconsin, Madison, WI.

2:00-2:45 p.m. **PANEL DISCUSSION**
Panel Chair: Deepak Bhatnagar

2:45-3:15 p.m. **BREAK**

SESSION 3: **CROP RESISTANCE — GENETIC ENGINEERING**
Chair: Lynn Jones. National Cottonseed Products Association

3:15 p.m. **"Engineering peanut for enhanced resistance to *Aspergillus flavus*."** Arthur K. Weissinger, North Carolina State University, Raleigh, NC.

3:30 p.m. **"Agrobacterium-mediated transformation and analysis of cotton expressing antifungal peptides."** Kanniah Rajasekaran,[1] Jeffrey W. Cary,[1] Anthony J. DeLucca,[1] Thomas J. Jacks,[1] Zhiyuan Chen[1], Alan R. Lax,[1] Caryl Chlan,[2] Jesse Jaynes,[3] and Thomas E. Cleveland.[1] [1]USDA, ARS, Southern Regional Research Center, New Orleans, LA; [2]University of Southwestern Louisiana, Lafayette, LA; and [3]Demeter Biotechnologies, Raleigh, NC.

3:45 p.m. **"Cotton transformation and a new system to assay efficacy of potential anti-fungal genes."** Caryl Chlan,[1] Jie Guo,[1] Kanniah Rajasekaran,[2] Jeffrey Cary,[2] Anthony J. Delucca,[2] and Thomas E. Cleveland.[2] [1]University of Southwestern Louisiana, Lafayette, LA and [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

4:00 p.m. **"Expression and inheritance of transgenes in peanut - greenhouse and field studies."** Peggy Ozias-Akins, University of Georgia, Tifton, GA

4:15 p.m. **"Genetic engineering of tree nut crops for control of aflatoxin."** Abhaya M. Dandekar, University of California, Davis, CA.

4:30 p.m. **"Anti-Aspergillus proteins: which ones work; which ones don't and why!"** Ray Bressan, Mike Hasegawa, Purdue University, West Lafayette, IN.

4:45 p.m. **"Lipoxygenase effects on *Aspergillus* development and aflatoxin production."** Nancy P. Keller, Texas A&M University, College Station, TX.

5:00-5:45 p.m. **PANEL DISCUSSION**
Panel Chair: Nancy P. Keller

5:45 p.m. — **POSTER VIEWING**

6:00 p.m. **COMMODITY BREAKOUT SESSIONS**

TUESDAY, OCTOBER 28, 1997

8:00 a.m. ANNOUNCEMENTS

8:15 a.m. INTRODUCTION OF SPEAKER: Bob Sacher, Hunt-Wesson

"Aflatoxins and the international scene." Sara H. Henry, FDA, Washington, D.C.

SESSION 4: **CROP RESISTANCE — CONVENTIONAL BREEDING**
Chair: John Green, National Corn Growers Association

8:45 a.m. **"Determination of maize kernel biochemical resistance to aflatoxin elaboration: mechanisms and new biotechnological tools."** Robert L. Brown,[1] Zhiyuan Chen,[2] Alan R. Lax,[1] Jeffrey W. Cary,[1] Thomas E. Cleveland,[1] John S. Russin,[2] Baozhu Z. Guo,[2] W. Paul Williams,[3] Georgia Davis,[3] Gary L. Windham,[3] and Gary A. Payne.[4] [1]USDA, ARS, Southern Regional Research Center, New Orleans, LA; [2]Louisiana State University, Baton Rouge, LA; [3]USDA, ARS, Mississippi State University, Mississippi State, MS; and [4]North Carolina State University, Raleigh, NC.

9:00 a.m. **"Evaluation of mutant B73 and A632 inbreds of corn for resistance to aflatoxin synthesis."** Charlie Martinson, Iowa State University, Ames, Iowa.

9:15 a.m. **"Inheritance of, molecular markers associated with, and breeding for, resistance to *Aspergillus* ear rot and aflatoxin production in corn using Tex6."** Donald G. White, Torbert R. Rocheford, Andrew M. Hamblin and Amy M. Forbes, University of Illinois, Urbana, Illinois.

9:30 a.m. **"Aflatoxin and BGYF kernels in a commercial corn hybrid inoculated with *Aspergillus flavus* genotypes isolated from corn in Illinois."** Donald T. Wicklow, USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

9:45 a.m. **"Results from screening the peanut core collection for resistance to preharvest aflatoxin contamination."** C. Corley Holbrook,[1] David M. Wilson,[2] and Michael E. Matheron.[3] [1]USDA, ARS, Coastal Plains Exp. Station, Tifton, GA; [2]University of Georgia, Coastal Experiment Station, Tifton, GA; and [3]University of Arizona, Yuma, AZ.

10:00 a.m. **"Characterization and field testing of an integrated fungal pathogen, insect vector resistance to aflatoxin contamination of almond."** Thomas M. Gradziel, University of California, Davis, CA.

10:15-10:45 a.m. **BREAK**

10:45 a.m. **"Characterization of inhibitors from corn seeds and the use of a new reporter construct to select corn genotypes resistant to aflatoxin accumulation."** Gary A. Payne, North Carolina State University, Raleigh, NC.

11:00 a.m. **"Aflatoxin-inducing metabolites in maize kernels: are they potential targets for aflatoxin elimination?"** Charles P. Woloshuk, Purdue University, West Lafayette, IN.

11:15 a.m. **"Studies on the identification of resistance to aflatoxin contamination of corn using an *Aspergillus parasiticus* mutant."** David M. Wilson,[1] Jason H. Brock,[1] and Neil W. Widstrom.[2] [1]University of Georgia, Coastal Experiment Station, Tifton, GA and [2]USDA, ARS, Insect Biology and Population Management Research Lab., Tifton, GA.

11:30-12:15 p.m. **PANEL DISCUSSION**
Panel Chair: Charles P. Woloshuk

12:15-1:30 p.m. **LUNCH**

SESSION 5: **MICROBIAL ECOLOGY**
Chair: Phillip Wakelyn, National Cotton Council

1:30 p.m. **"Obstacles to the use of *Aspergillus flavus* to prevent aflatoxin contamination in commercial agriculture."** Peter Cotty, USDA, ARS, Southern Regional Research Center, New Orleans, LA.

1:45 p.m. **"Potential for biological control of preharvest aflatoxin contamination of corn using competitive, nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus*."** Joe W. Dorner,[1] Richard J. Cole,[1] and Donald T. Wicklow.[2] [1]USDA, ARS, National Peanut Research Laboratory, Dawson, GA and [2]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

2:00 p.m. **"Geographic information systems (GIS) reveal recurring patterns of S and L forms of *Aspergillus flavus*."** Merritt R. Nelson,[1] Donna M. Bigelow,[1] Peter J. Cotty,[2] and Thomas V. Orum.[1] [1]University of Arizona, Tucson, AZ and [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

2:15 p.m. **"Soil populations of *Aspergillus* species from section Flavi along a transect through peanut-growing regions of the United States."** Bruce W. Horn, R. Larry Greene, and Joe W. Dorner, USDA, ARS, National Peanut Research Laboratory, Dawson, GA.

2:30-3:15 p.m. **PANEL DISCUSSION**
Panel Chair: Joe W. Dorner

3:15-3:30 p.m. **CLOSING REMARKS:** Jane F. Robens
NEXT MEETING

3:30 p.m. **AFLATOXIN TECHNICAL GROUP MEETING**

POSTER PRESENTATIONS
1997 Aflatoxin Elimination Workshop
October 26-28, 1997
Memphis, TN

Poster Board Number

(1) A. EXTRACTION, DETECTION AND ANALYSIS OF AFLATOXINS

2 A-1 **"The use of supercritical fluids for the extraction and analysis of aflatoxins."** Scott L. Taylor,[1] Jerry W. King,[1] Judith I. Greer,[1] and John L. Richard.[2] [1]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL and [2]Romer Labs, Inc., Union, MO.

3 A-2 **"A rapid fluorometric test for the quantitation of aflatoxins in corn, nuts and cottonseed."** Bruce R. Malone, Tom R. Romer, John L. Richard and Craig W. Humphrey, Romer Labs, Inc., Union, MO.

(4) B. MICROBIAL ECOLOGY

5 B-1 **"Spread of a genetically altered *Aspergillus* isolate in maize ears in the field."** Gary L. Windham,[1] W. Paul Williams,[1] Robert L. Brown,[2] Thomas E. Cleveland,[2] and Gary A. Payne.[3] [1]USDA, ARS, Mississippi State University, Mississippi State, MS; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA; and [3]North Carolina State University, Raleigh, NC.

6 B-2 **"Genotypic diversity of *Aspergillus parasiticus* in an Illinois corn field."** Cesaria E. McAlpin, Donald T. Wicklow, and Crystal E. Platis, USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

7 B-3 **"Interactions of saprophytic yeasts with *Aspergillus flavus* in wounded pistachios."** Sui-Sheng T. Hua, James L. Baker, Ok-Koo Grosjean, and Melanie Flores-Espiritu, USDA, ARS, Western Regional Research Center, Albany, CA.

(8) C. CROP MANAGEMENT AND HANDLING. INSECT CONTROL AND FUNGAL RELATIONSHIPS

9 C-1 **"Segmentation and classification of pistachio nuts using linescan x-ray data."** David Casasent,[1] Ashit Talukder,[1] Ha-Woon Lee,[1] Pamela M. Keagy[2] and Tom F. Schatzki.[2] [1]Carnegie Mellon University, Pittsburg, PA and [2]USDA. ARS, Western Regional Research Center, Albany, CA.

10 C-2 **"Detection of insect damage in almonds by image processing of x-ray images."** Pamela M. Keagy and Ray Miller, USDA. ARS, Western Regional Research Center, Albany, CA.

11 C-3 **"Activity of a novel corn kernel protein against insects."** Patrick F. Dowd,[1] Ashwin Mehta[2] and Rebecca Boston.[2] [1]USDA. ARS. National Center for Agricultural Utilization Research, Peoria, IL and [2]North Carolina State University, Raleigh, NC.

12 C-4 **"Aflatoxin accumulation in transgenic Bt corn hybrids after insect infestation."** W. Paul Williams, Gary L. Windham and Frank M. Davis, USDA. ARS, Mississippi State University, Mississippi State, MS.

13 C-5 **"Aflatoxin contamination of Bt cottonseed in commercial fields and in field plots."** Peter J. Cotty,[1] Don R. Howell,[2] Clive H. Bock,[1] and Alfonso Tellez.[2] [1]USDA. ARS, Southern Regional Research Center, New Orleans, LA and [2]Cooperative Extension Service, University of Arizona, Yuma, AZ.

14 C-6 **"Influence of harvest date on aflatoxin contamination in western Arizona in 1995 and 1996."** Clive H. Bock and Peter J. Cotty, USDA. ARS, Southern Regional Research Center, New Orleans, LA

15 C-7 **"Influence of storage systems on aflatoxin contamination of maize in Africa."** Kitty F. Cardwell, Kerstein Hell, and Janet Udoh, Biological Control Center for Africa, International Institute of Tropical Agriculture, Cotonou, Benin.

16 C-8 **"Differences in the metabolic response of peanut plants to water stress."** Sheikh M. Basha, Florida A&M University, Tallahassee, FL.

(17) D. CROP RESISTANCE -- CONVENTIONAL BREEDING

18 D-1 **"Studies on the *Aspergillus parasiticus*: peanut interaction."** Frances Trail, Michigan State University, E. Lansing, MI.

19 D-2 **"*Aspergillus flavus* growth and aflatoxin accumulation in 15 maize isolines containing genes for anthocyanin production."** Georgia Davis, Gary L. Windham, and W. Paul Williams, USDA, ARS, Mississippi State University, Mississippi State, MS.

20 D-3 **"Antifungal proteins in corn kernels: immunochemical localization and induction during germination."** Baozhu Guo,[1] Zhiyuan Chen,[2] Robert L. Brown,[3] Alan R. Lax,[3] Thomas E. Cleveland,[3] John S. Russin,[2] and Neil W. Widstrom.[1] [1]USDA, ARS, Insect Biology and Population Mgmt. Research Lab., Tifton, GA.; [2]Louisiana State University, Baton Rouge, LA; and [3]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

21 D-4 **"Expression of the green fluorescent protein in *Aspergillus flavus* and its use as marker to evaluate resistance in corn kernels."** Wanglei Du and Gary A. Payne, North Carolina State University, Raleigh, NC.

22 D-5 **"Construction and analysis of an *Aspergillus flavus* omtA(p)::GUS reporter construct."** Carmen S. Brown-Jenco,[1] Gary A. Payne,[1] Robert L. Brown,[2] and Deepak Bhatnagar.[2] [1]North Carolina State University, Raleigh, NC and [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

(23) E. CROP RESISTANCE -- GENETIC ENGINEERING

24 E-1 **"Cloning and characterization of lipoxygenase gene(s) from peanut."** Gloria B. Burow,[1] Harold W. Gardner,[2] and Nancy P. Keller.[1] [1]Texas A&M University, College Station, TX and [2]USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

25 E-2 **"Use of Arabidopsis as a model system to test the *in vivo* efficacy of gene products against *Aspergillus flavus*."** Caryl Chlan,[1] Jie Guo,[1] Kanniah Rajasekaran,[2] Jeffrey W. Cary,[2] Anthony J. Delucca,[2] and Thomas E. Cleveland.[2] [1]University of Southwestern Louisiana, Lafayette, LA and [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

26 E-3 **"Transformation and analysis of cotton and tobacco tissues expressing antifungal proteins and peptides."** Jeffrey W. Cary,[1] Kanniah Rajasekaran,[1] Anthony J. Delucca,[1] Thomas J. Jacks,[1] Zhiyuan Chen,[2] Alan R. Lax,[1] Caryl Chlan,[3] Jesse Jaynes,[4] and Thomas E. Cleveland.[1] [1]USDA, ARS, Southern Regional Research Center, New Orleans, LA; [2]Louisiana State University, Baton Rouge, LA; [3]University of Southwestern Louisiana, Lafayette, LA; and [4]Demeter Biotechnologies, Raleigh, NC.

27 E-4 **"Purification of a small peptide with antifungal activity against *Aspergillus flavus*."** Anne L. Moynel,[1] Thomas E. Cleveland[2] and Sadek Tuzunl[1]. [1]Department of Plant Pathology, Auburn University, Auburn, AL and [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA

(28) F. **REGULATION OF AFLATOXIN BIOSYNTHESIS**

29 F-1 **"Molecular characterization of the effects of nutritional factors on transcription and aflatoxin production by reporter strains of *Aspergillus parasiticus*."** Matthew Rarick, Michael Miller, and John E. Linz, Michigan State University, E. Lansing, MI..

30 F-2 **"Immunodetection of gene products involved in aflatoxin biosynthesis in *Aspergillus parasiticus*."** Ching-Hsun Chiou, Li-Wei Lee, and John E. Linz, Michigan State University, E. Lansing, MI.

31 F-3 **"Characterization of a regulatory protein, AFLR, in *Aspergilli*."** Biing Hui Liu and Fun S. Chu, University of Wisconsin, Madison, WI.

32 F-4 **"Is an AFLR-repressor interaction involved in nitrate inhibition of aflatoxin biosynthesis in *Aspergillus parasiticus*?"** Perng-Kuang Chang,[1] Jiujiang Yu,[2] Deepak Bhatnagar,[2] Joan W. Bennett,[1] and Thomas E. Cleveland.[2] [1]Tulane University, New Orleans, LA and [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

33 F-5 **"Site-directed mutagenesis of the aflatoxin biosynthesis regulatory gene, *aflR*."** Kenneth C. Ehrlich, Beverly Montalbano, Deepak Bhatnagar and Thomas E. Cleveland, USDA, ARS, Southern Regional Research Center, New Orleans, LA.

34 F-6 **"Molecular and genetic analyses of aflatoxin regulatory mutants in *Aspergillus flavus*."** Matthew P. Brown and Gary A. Payne, North Carolina State University, Raleigh, NC.

35 F-7 **"Insights into regulation of aflatoxin biosynthesis from an unusual *Aspergillus flavus* strain from Africa."** Peter J. Cotty,[1] Toby Feibelman,[1] and Kitty F. Cardwell.[2] [1]USDA, ARS, Southern Regional Research Center, New Orleans, LA and [2]Biological Control Center for Africa, International Institute of Tropical Agriculture, Cotonou, Benin.

36 F-8 **"Use of site-directed mutagenesis for determination of amino acid residues critical for oxidoreductase activity involved in aflatoxin biosynthesis."** Jiujiang Yu,[1] Kenneth C. Ehrlich,[1] Perng-Kuang Chang,[2] Deepak Bhatnagar,[1] and Thomas E. Cleveland.[1] [1]USDA, ARS, Southern Regional Research Center, New Orleans, LA and [2]Tulane University, New Orleans, LA.

(37) G. **POTENTIAL USE OF NATURAL PRODUCTS FOR PREVENTION OF FUNGAL INVASION AND/OR REGULATION OF AFLATOXIN BIOSYNTHESIS IN CROPS**

38 G-1 **"Plant metabolites alter *Aspergillus* development."** Ana M. Calvo,[1] Lori L. Hinze,[1] Harold W. Gardner,[2] and Nancy P. Keller.[1] [1]Texas A&M University, College Station, TX and [2]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

39 G-2 **"Effect of walnut constituents on *Aspergillus* growth and aflatoxin production."** Noreen Mahoney,[1] Russell J. Molyneux,[1] and Gale McGranahan.[2] [1]USDA, ARS, Western Regional Research Center, Albany, CA and [2]University of California, Davis, CA.

40 G-3 **"Host-Plant volatiles preferentially enhance the attractancy of the sex pheromones of moth pests associated with *Aspergillus* infection of tree nuts."** Douglas M. Light and Christopher N. Mehelis, USDA, ARS, Western Regional Research Center, Albany, CA.

41 G-4 **"Inhibition of Gus Reporter gene of aflatoxin biosynthesis by plant signal molecules."** Sui-Sheng T. Hua, Melanie Flores-Espiritu, Albert Hong, James L. Baker and Ok-Koo Grosjean, USDA, ARS, Western Regional Research Center, Albany, CA.

42 G-5 **"A maize kernel trypsin inhibitor is associated with resistance to *Aspergillus flavus* infection."** Zhiyuan Chen,[1] Robert L. Brown,[2] Alan R. Lax,[2] Baozhu Guo,[3] Thomas E. Cleveland,[2] and John S. Russin.[1] [1]Louisiana State University, Baton Rouge, LA; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA; and [3]USDA, ARS, Insect Biology and Population Management Research Lab, Tifton, GA.

43 G-6 **"Potential role for storage proteins and sugars in oilseed susceptibility to aflatoxin contamination."** Jay E. Mellon and Peter J. Cotty, USDA, ARS, Southern Regional Research Center, New Orleans, LA.

44 G-7 **"β-carotene inhibition of aflatoxin biosynthesis by *Aspergillus flavus* genotypes isolated from Illinois corn."** Donald T. Wicklow, Robert A. Norton, and Cesaria E. McAlpin, USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

45 G-8 **"Characterization of an alpha-amylase deficient mutant of *Aspergillus flavus*."** Ahmad Fakhoury and Charles P. Woloshuk, Purdue University, West Lafayette, IN.

46 G-9 **"Inhibitory effects of volatile aldehydes from aflatoxin-resistant varieties of corn on *Aspergillus parasiticus* growth and aflatoxin biosynthesis."** Maureen S. Wright, Dana M. Greene-McDowelle, Hampden J. Zeringue, Jr., Deepak Bhatnagar, and Thomas E. Cleveland, USDA, ARS, Southern Regional Research Center, New Orleans, LA.

47 G-10 **Characterization of a chitinase from Tex6 inhibitory to *Aspergillus flavus*."** Gary A. Payne,[1] and Donald G. White.[2] [1]North Carolina State University, Raleigh, NC and [2]University of Illinois, Urbana, IL.

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(Poster Board numbers and the subject category are listed for each author.)

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